IMPERIAL INSTITUTE

OF

AGRICULTURAL RESEARCH, PUSA.

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milk, a technique previously standardized for these studies and reported earlier in this *Journal* (6, 8), was followed. Young rats approximately 21 days old were placed in individual cages on raised screen bottoms in which cups of vitamin B-free ration were available at all times. This diet consists of 18 per cent casein,[2]

TABLE I

Case Histories of Women

	Subject VI	Subject VII	Subject VIII
Descent.................	Dutch, French, German, Danish	Scotch, Irish, American	Dutch, French, German, Danish
Age, *yrs*................	25	33	27
Height, *in*..............	62.2	68.5	62.6
Body weight, *kg.*			
Beginning lactation....	73.1	64.9	70.4
End "	65.8	61.7	81.7
Usual physical state.....	Apparently healthy, persistently low hemoglobin	Healthy, no defects	Apparently healthy, albuminuria
No. of pregnancies.......	4	3	3
Date of birth of babies...	Aug. 20, 1928	Sept. 14, 1928	July 17, 1928
Weight of infant at birth..	8 lbs., 10 oz.	9 lbs., 3 oz.	6 lbs., 12 oz.
Development of infant..	Excellent	Excellent	Excellent
Average output of milk per 24 hrs., *cc*..........	3134	2366	1419

76 per cent dextrin, 4 per cent salt mixture,[3] and 2 per cent agar-agar, together with 5 drops of cod liver oil[4] and 2 drops of wheat germ oil to supply the vitamins A, D, and E. The breast milk served as the only source of vitamin B. The vitamins and milk were fed fresh daily in measured quantities in separate dishes

apart from the basal ration. Weekly growth records and food intake were determined.

Rats do not take all human milks with the same degree of relish, and so in certain cases do not take the entire amount of milk pro-
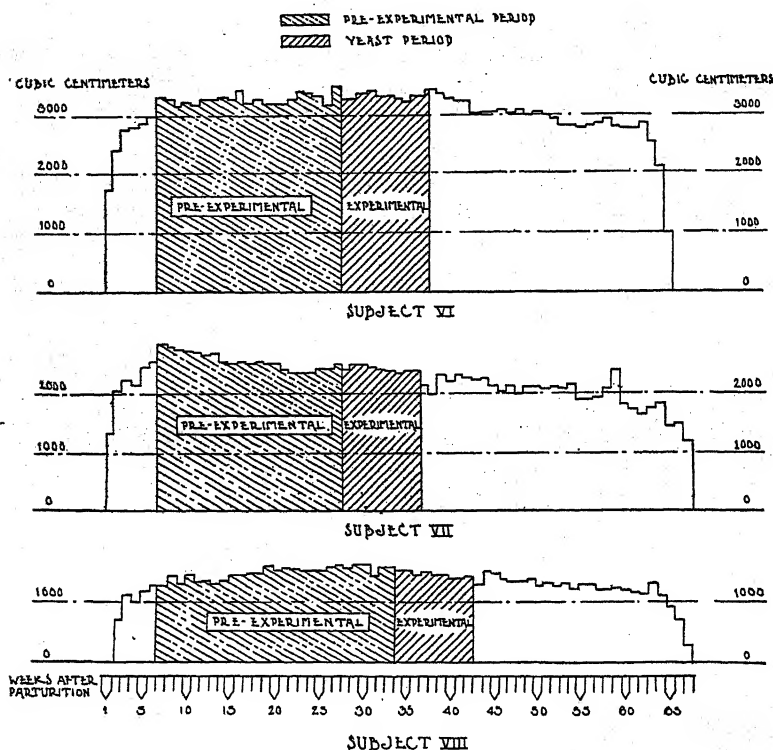
CHART I. Average daily quantity of milk secreted by three women throughout a lactation cycle of 14 months. The quantity output remained practically constant during the period preceding and during the consumption of 10 gm. of yeast daily.

vided. In this investigation only the animals taking the complete daily allotments of milk are considered.

After the vitamin B studies on the milk had progressed for a period of 6 weeks, 0.4 gm. of autoclaved yeast[5] was added to the

daily dietary of the rat to determine whether the absence of vitamin G was a limiting factor in breast milk (6, 16).   The animals were continued on the autoclaved yeast for a period of 3 weeks.

EXPERIMENTAL

It has been shown that the relative vitamin B content of breast milk can be determined by feeding to rats levels of 16, 20, and 25 cc. of the milk daily in conjunction with a diet that is complete
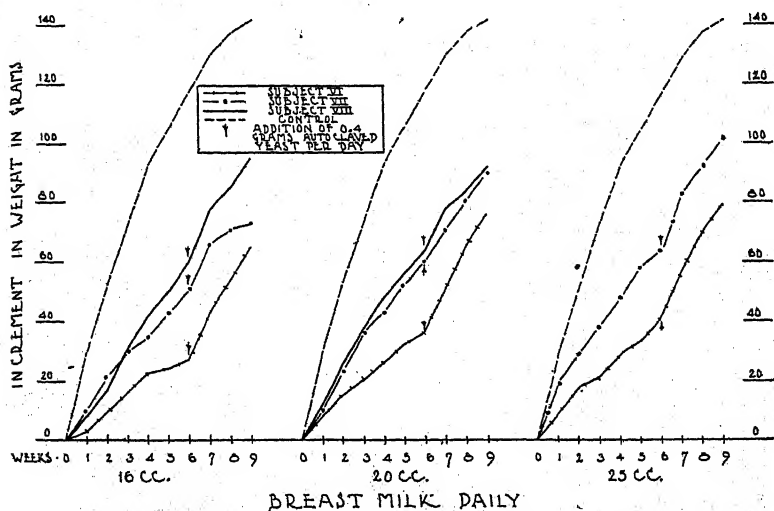
CHART II.   Comparison of average gains in weight of three series of rats on breast milks from three different women on their accustomed diets, fed at levels of 16, 20, and 25 cc. daily for 6 weeks as the sole source of vitamin B. At the end of this period 0.4 gm. of autoclaved yeast was fed daily for 3 weeks.

in known dietary constituents with the exception of vitamin B (5).   Since the milk output of the women was not in great excess of the amount the babies took, it was not possible to carry more than five animals on each level of the individual milks.

Chart I shows the level of milk production and its duration for the three subjects in terms of the daily average quantity of milk expressed during each week throughout the lactation cycle.   The period of assaying the milk for vitamin B before supplementing the usual diets is indicated as *preexperimental*; the period during which

the usual diets were supplemented with 10 gm. of yeast daily as *experimental.* The constancy of milk output throughout the two periods shows that for these particular subjects the quantity of the secretion was apparently not affected by the vitamin-containing supplements to the diets.

Chart II illustrates the average gain in weight of the three series of rats during the preexperimental period, when breast milk from the three individual women was fed at levels of 16, 20, and 25 cc. daily and served as the sole source of vitamin B. The differences
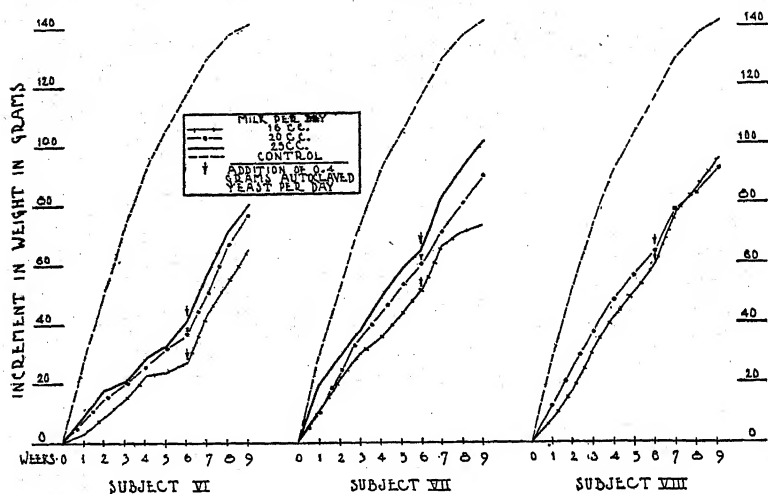
CHART III. Comparison of average gains in weight of three series of rats fed the same daily levels of milk from three different women on their accustomed diets, as the sole source of vitamin B during 6 weeks. At the end of this period 0.4 gm. of autoclaved yeast was fed daily for 3 weeks.

in the rate of growth of the three series of animals on the different milks demonstrate that there is a continuous difference in the vitamin potency of the milks of the three women (Chart III). It has been suggested in a previous publication (7) that there is a possible relation between the total volume of milk secreted and vitamin B potency of the milk. The present investigation substantiates this inference. Subject VI had an average daily output of over 3134 cc. of milk daily over a period of 14 months, during the same interval Subject VII produced 2366 cc. daily, and Subject VIII 1419 cc. daily. The gradation in vitamin potency of

the three milks was in accordance with the volume of milk pro-
duced; *i.e.*, the milk of Subject VI was lowest in vitamin B content,
the milk of Subject VII next highest, and that of Subject VIII
the most potent of the three.   These findings may indicate either
a dilution phenomenon or a greater demand for vitamin B in the
actual physiological protoplasmatic processes involved in the
elaboration of large quantities of milk.

The food intakes of the three women average between 3900 and
4600 calories daily; the protein from 162 to 168 gm. daily.   The
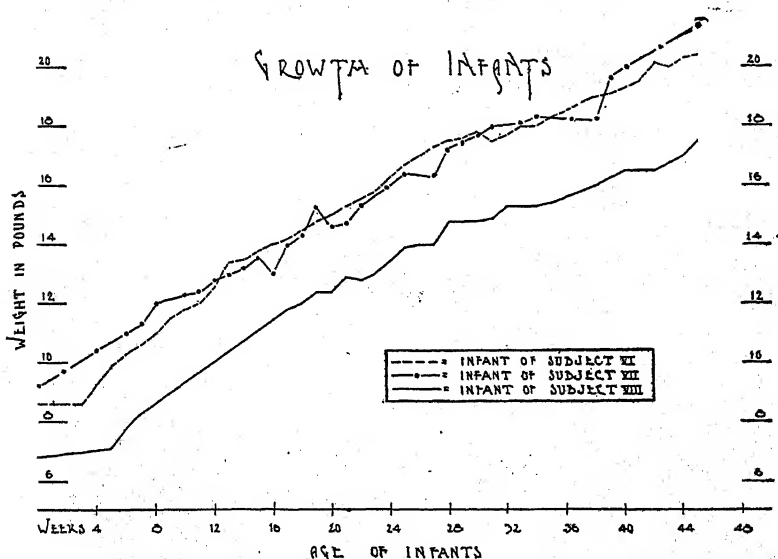
CHART IV. Growth of infants of Subjects VI, VII, and VIII over the
first 45 weeks of life.   All infants gained approximately 4 ounces per week.

food consumption records were based upon weighed dietaries on
5 and 10 consecutive day studies at different intervals in the lacta-
tion period.   It has been shown in a previous publication (17)
that "Subjects VI and VIII ate more eggs and cheese than Subject
VII.   Subject VIII used very little fruit as compared to Subjects
VI and VII.   Subject VII had a large consumption of bread and
other cereals.   Subject VI ate more meat than either Subjects
VII or VIII."

The birth weights of the infants of Subjects VI, VII, and VIII

were 8 pounds 10 ounces, 9 pounds 3 ounces, and 6 pounds 12 ounces, respectively.   The babies all grew at the rate of approximately 4 ounces per week during the first 45 weeks of life; during
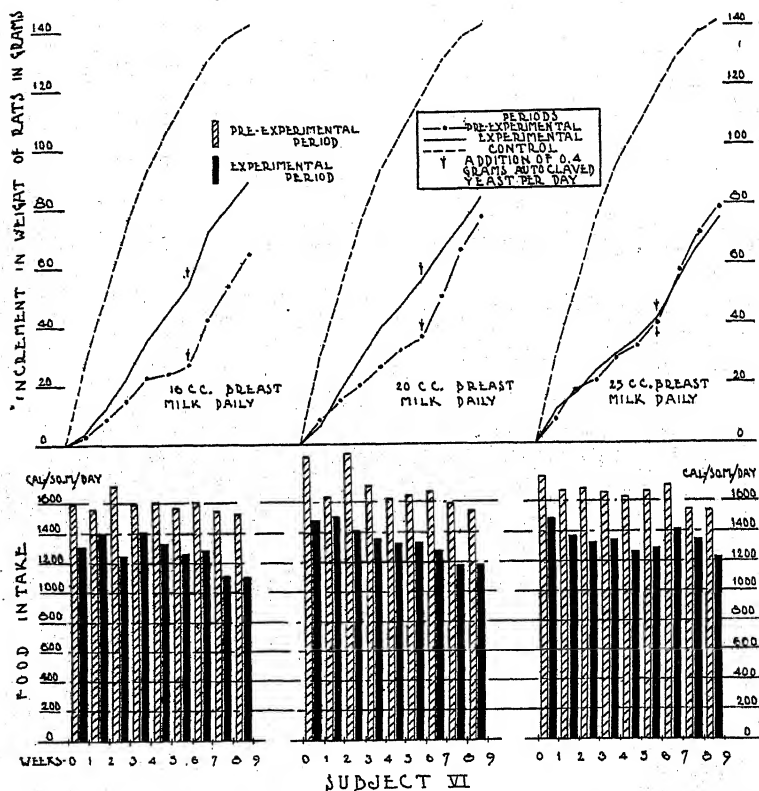
CHART V.   Average gain in weight and food intake of groups of rats fed on levels of 16, 20, and 25 cc. of the milk of Subject VI as the sole source of vitamin B.   The dot and dash line represents the growth of the rats when Subject VI was consuming her accustomed home diet.   The solid line represents their growth when her diet was supplemented with 10 gm. of yeast daily.   The average weekly food intake in calories per sq. m. of body surface per 24 hours is shown for each period and for each level of milk.

the first 26 weeks they were given little food other than their mothers' milk (Chart IV).   The growth of all three infants was within the standards accepted by pediatricians.

The preexperimental period extended from the 6th week to the

6th month of lactation.   During this time the three subjects ate their accustomed diets, uncontrolled in quantity and quality.   The milks secreted during this period were each tested for vitamin B
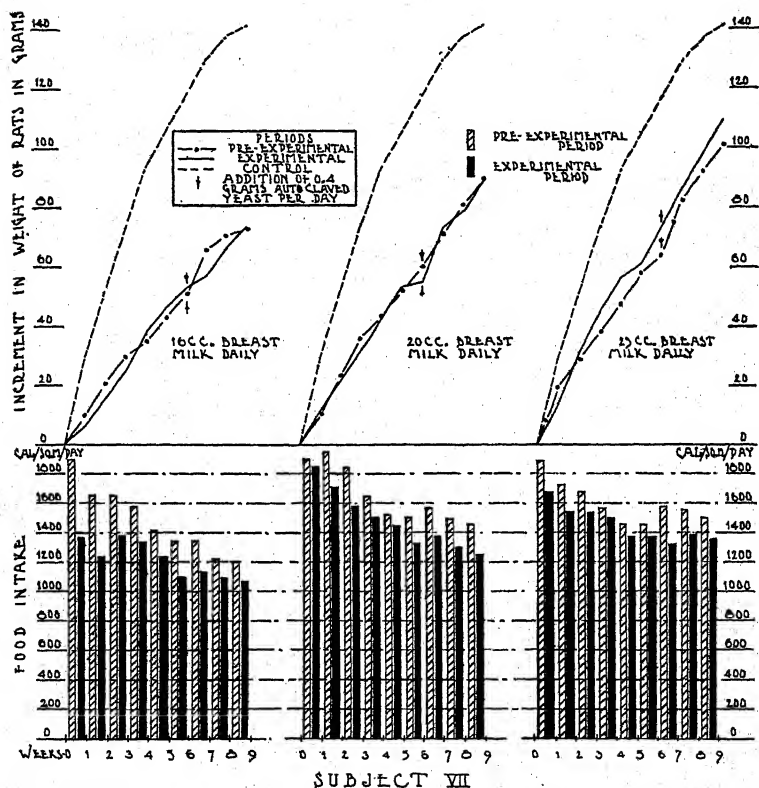
CHART VI.   Average gain in weight and food intake of groups of rats fed on levels of 16, 20, and 25 cc. of the milk of Subject VII as the sole source of vitamin B.   The dot and dash line represents the growth of the rats when Subject VII was consuming her accustomed home diet.   The solid line represents their growth when her diet was supplemented with 10 gm. of yeast daily.   The average weekly food intake in calories per sq. m. of body surface per 24 hours is shown for each period and for each level of milk.

potency upon rats under standardized conditions.   The experimental period extended from the 6th to the 10th month of the lactation period.   During this time each of the women took 10 gm. of

yeast daily, and each of the milks was again tested for vitamin B concentration.

Chart V shows the growth of the animals during the preexperimental and experimental periods when the milk of Subject VI served as the sole source of vitamin B; Chart VI shows the same data for Subject VII; and Chart VII the same data for Subject VIII. It is evident from these graphs that in general each of the three series of animals grew at approximately the same rate during the preexperimental and experimental periods; in the instances where the rate of growth differed during the two periods, it was appreciably better during the experimental period. The most striking observation to be made here concerns the average caloric intake per square meter of surface area of the rats[6] during a 24 hour period of the preexperimental period on each level of milk from each of the three women, as contrasted with the average caloric intake during a like interval of the experimental period. It is significant that in all cases a greater number of calories were consumed during the preexperimental period than during the experimental period to produce the same amount of growth and in some cases less growth. In other words, some substance was carried over into the milk from the maternal diet supplemented daily with 10 gm. of yeast which stimulated better utilization of the food consumed by the experimental animals. It is obvious that this substance is not an appetite stimulator; it is consequently improbable that it is the antineuritic vitamin. On the other hand, some other food factor from the maternal diet, other than that of the appetite-stimulating substance, is carried over into the breast milk, and this factor is responsible for a more economical and effective use of the food material in building the body of the experimental rat than is obtained without it. In other words, the animals consuming milk from a mother who is taking yeast receive a dietary component that promotes the elaboration of body tissues at a more effective rate on a smaller amount of food.

The total calorie intake of the animals on levels of 16, 20, and 25 cc. respectively of breast milk for each of the three subjects during the preexperimental period as compared with the experi-

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[6] The surface area of the rats was calculated according to the formula used by Meeh, with the constant 9.1 as suggested by Rubner (18).

mental period showed not only that there is a greater intake in all cases during the preexperimental period than during the experimental period; but also that the quantity of basal food taken by
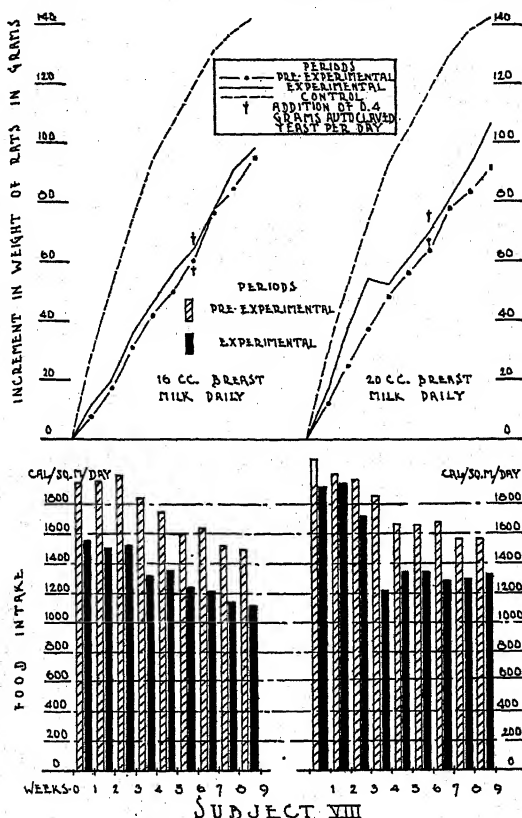
CHART VII. Average gain in weight and food intake of groups of rats fed at levels of 16 and 20 cc. of the milk of Subject VIII as the sole source of vitamin B. The dot and dash line represents the growth of the rats when Subject VIII was consuming her accustomed home diet. The solid line represents their growth when her diet was supplemented with 10 gm. of yeast daily. The average weekly food intake in calories per sq. m. of body surface per 24 hours is shown for each period and for each level of milk.

the three series of animals during the same period varies with the different milks. Thus there is a constant increase in food intake on the milks of Subjects VI, VII, and VIII respectively. This is

in accord with the vitamin B content of the milks of the three women.   In all cases the animals receiving the milk of Subject VIII, which is richest in vitamin B, had a greater basal food intake during both periods than did the animal receiving a like quantity of the milks of Subject VI or Subject VII during the same periods.

At the end of the 6 weeks of the vitamin test period, 0.4 gm. of autoclaved yeast, known to be a rich source of vitamin G, was superimposed daily upon the diets of the experimental animals. In most cases, especially with the milk from the preexperimental periods and the milk with a lower concentration of vitamin B, the rats responded with a stimulated increase in growth.   This is in accord with previous findings in these laboratories (16).   Further consideration is being given to this phase of the studies.

SUMMARY

1. The breast milks from three women were each tested separately on rats for their vitamin B content.   The potencies were determined before and after supplementing the usual maternal diets with 10 gm. of yeast daily.

2. A comparison showed the vitamin B content of the three milks to be inversely proportional to the quantity of milk secreted daily.

3. The results for these three subjects indicate that some substance was carried over into the milk from the maternal diet containing daily supplements of 10 gm. of yeast which stimulated more economical utilization of the food consumed by the experimental animals.   This substance did not stimulate the appetite, consequently food consumption was not augmented.

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METHOD FOR THE ESTIMATION OF ENZYME YIELD IN FUNGUS CULTURES

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The following paper presents a new method for the determination of the enzyme yield of mold cultures and for obtaining comparable enzyme determinations on solid and liquid materials. As an example the method will be applied to the saccharase of *Penicillium glaucum,* and data will be presented to show the significance and suitability of the method.

Fernbach (1890) proposed a method (7) and a unit for the estimation of saccharase. He defines the unit of saccharase as the quantity of enzyme which is able to hydrolyze 20 centigrams of sucrose in 1 hour in the presence of 0.01 per cent acetic acid and at 56°. In his studies on *Aspergillus niger* and on brewers' yeast he used this method to establish the intensity of the saccharase effect. He first determined the enzyme content of the medium of the mold cultures. Then he made an extract of the ground mold to the same volume as the culture medium had been and determined the saccharase content of this extract. He compared these results (expressed in saccharase units), and to obtain the total amount of enzyme he added them. At that time this method was of great importance, not only because it suggested a procedure for the determination of enzyme activity, but also because it proposed a definite unit for measuring and expressing these activities. But later work showed it to have serious defects. The essential mistake was that he did not determine the enzyme in the mold directly, but in a solution made up from the killed cells, and set down this result as the enzyme content of the mold. But von Euler and Svanberg (5), working with yeast, established that the solution made according to Fernbach's direction did not contain

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more than 90 per cent of the total amount of saccharase, depending on the concentrations and on the temperature of the extraction. Although the great importance of Fernbach's idea is generally conceded, his method is no longer used for the determination of the enzyme content of a plant or of an enzyme preparation. Since the work of Fernbach, no general method has been proposed for the determination of the total enzyme yield of mold or bacterial cultures. The different enzyme units, time values, and measurements of the enzyme activity on the basis of the velocity constant k need not be discussed here. All of these methods show the enzyme activity of a material, but cannot be used for expressing the total enzyme yield.

In the case of a monomolecular enzyme reaction the well known velocity constant is

$$k = \frac{1}{t \times 0.4343} \log \frac{A}{A - X}$$

in which equation t is the reaction time in minutes, A is the amount of substrate present at the beginning of the reaction, and X is the amount of substrate changed in time t. To obtain comparable values it is necessary to use the natural logarithms, or the factor 0.4343, and Briggs' logarithms, as in the above formula. The k in one experimental series is proportional to the enzyme activity, but only if all experimental conditions, such as amount of enzyme and substrate, pH, and temperature were constant except one, which changes. If it is assumed that the catalytic effect of the enzyme is independent of the quantity of the substrate present, then the following expression for the enzyme activity may be derived.

$$\text{Enzyme activity } (Xf) = \frac{k}{\text{gm. of dry matter used}}$$

The foregoing formula, which was proposed by von Euler and collaborators (4), is a generalization of the earlier formula which was proposed by von Euler and Svanberg (6) to characterize the effect of the saccharase preparations.

$$If = \frac{k \times \text{gm. of sucrose}}{\text{gm. of dry matter used}}$$

in which *If* is "Inversionsfähigkeit," or hydrolytic activity. The gm. of sucrose means the actual gm. of sucrose present in the reaction mixture at the beginning of the experiment. The dry matter means the amount of dry matter contained in the enzyme preparation present in the reaction. The significance of the *If* value is, of course, limited to a certain range of concentrations, and it must be determined at the optimal pH and temperature. This formula, which is now commonly used, expresses the catalytic power of a dry matter unit (gm.) of the enzyme preparation. By multiplying by the total dry weight of the mold or tissue the total yield of enzyme is obtained, as was proposed by the writer in 1927.[1]

Then by combining the various equations we have:

$$E_1 = \frac{k \times \text{gm. of sucrose}}{\text{gm. of mycelium used}} \times \text{total mycelium (dry, in gm.)}$$

Thus E_1 is the total yield of saccharase in the mycelium. E_2 can in the same way be the total yield of saccharase in the medium, by substituting cc. of medium for gm. of mycelium in both places. Then $E_1 + E_2 =$ total yield of enzyme in the culture as grown.

In the case of the medium, one precaution is necessary. In calculating k, the A must include not only the sucrose added to the reaction mixture, but also the sucrose contained in the medium used in the determination.

EXPERIMENTAL

The mold cultures (*Penicillium glaucum*, Link), unless otherwise noted, were always grown at 24° on the following medium.

	per cent
KH_2PO_4	0.100
$CaCl_2$	0.010
$MgSO_4$	0.030
NaCl	0.010
$FeCl_3$	0.001
$(H_4N)NO_3$	0.100

In addition to the salt mixture sucrose was added to the medium in varying amounts. In the cultures grown in the absence of

[1] A lecture that was given before the Chemical Faculty of the Royal Hungarian Society of Natural Science, January 25, 1927.

1 ion from the given salt supplement, the absent ion was replaced by an equivalent amount of another ion. The determination of the saccharase effect was carried out at pH 4.5, which has been shown to be the optimum for this enzyme (8), and at 38.0° in the presence of 3 per cent of toluene. The sucrose concentration of the reaction mixture was 5 per cent. The mycelium was applied as a suspension, after being washed free from the medium and ground. Earlier investigations (3), which were confirmed by Doby and Feher (1), also showed that while the value of the k decreases considerably in the 1st hours, after the 5th hour it is roughly constant till the reaction reaches a very advanced stage. Therefore, the values of k were calculated from the polarimeter readings beginning with the 6th hour. The readings were made in a 100 mm. tube, after the sample was clarified by lead acetate and sodium carbonate solutions. Further experimental details are given in the work of Doby and Kertesz (3).

Relation between If and Gm. of Yield (Dry Matter)

It will be seen that E_1 is practically constant for all cultures of the same sucrose content, but that it increases with an increase in the sucrose content. It is surprizing that E_1 is so constant, since the yield of mycelium in any one group may vary 100 per cent. However, the yield and the k vary conversely, and their product is a constant.

In Table I are collected the results, obtained by the present writer, as well as some of those by Doby and Feher, on the effect of growing the mold on different concentrations of sucrose. The volume of medium and the age of the cultures was the same in all cases.

The experiments given in Table I were performed on cultures with complete salt supplement. In the work of Doby and Kertesz and in that of Doby and Feher, data were presented to show the shape assumed by the E_1 curve if certain important ions were omitted from the media, with the sucrose nourishment varied. Table II and Fig. 1 show the E_1 values when the cultures were deprived of potassium, phosphorus, magnesium, or calcium, except very small amounts of phosphorus, without which no growth could be obtained.

TABLE I

Relation between "If" and Yield of Mycelium in Mold Cultures

Grown for 5 days, on 250 cc. of sucrose solution, with complete salt supplement.

Sucrose in medium, per cent	5	5	5*	10	10*	10*	10*	10*	15	15*	20*	20*	20*	20*	20*	20*	20*	20*
Dry matter yield, gm.	0.53	0.62	0.70	0.78	0.61	0.69	1.20	0.96	0.92	0.80	0.77	0.56	0.79	0.78	0.72	0.63	0.91	0.48
$If \times 10^3$	31.6	23.3	29.9	35.7	44.7	40.8	22.4	26.8	38.6	44.4	55.9	74.0	61.0	57.8	64.4	64.4	45.3	80.8
$E_1 \times 10^3$	16.8	14.5	16.0	27.8	26.8	28.4	26.8	26.6	35.5	35.5	43.0	41.4	48.1	45.0	46.2	40.5	41.1	38.7
$E_1 \times 10^3$, average.			15.8					27.3		35.5								43.0

* Data taken from Doby and Feher's work (1).

TABLE II

E_1 *in Penicillium glaucum Cultures Growing with 1 Ion Absent from Salt Supplement*

5 days on 250 cc. of medium, at 24°.

Sucrose in medium	Complete (see Table I) $E \times 10^3$	Without K*			Without Ca†			Without Mg†			With 0.001 per cent P†			With 0.0002 per cent P†		
		Dry matter yield	$If \times 10^3$	$E \times 10^3$	Dry matter yield	$If \times 10^3$	$E \times 10^3$	Dry matter yield	$If \times 10^3$	$E \times 10^3$	Dry matter yield	$If \times 10^3$	$E \times 10^3$	Dry matter yield	$If \times 10^3$	$E \times 10^3$
per cent																
2.5	4.1	0.13	57.2	7.43	0.44	22.1	8.5	0.02	52.2	0.85	0.24	14.8	5.1	0.05	79.4	3.9
5	15.8	0.13	41.8	5.43	0.57	25.2	14.3	0.04	38.6	1.5	0.36	23.6	8.9	0.10	68.3	6.8
10	27.3	0.22	19.5	4.29	0.64	37.9	24.7	0.10	28.6	2.8	0.45	36.6	16.4	0.13	53.6	7.1
15	35.5	0.33	15.7	5.18	0.76	43.5	33.0	0.15	21.1	3.1	0.40	44.4	17.8	0.17	44.5	8.6
20	43.0	0.42	10.1	4.24	0.71	55.1	39.3	0.22	14.5	3.6	0.47	41.3	19.9	0.20	35.1	8.8

* From Doby and Kertesz (3).
† From Doby and Feher (1).

E_1 of Penicillium glaucum Cultures of Different Salt Supplements

In completely nourished cultures the value of E_1 rises with increasing sucrose nourishment, as was noted in Table I. In the cultures where K or Mg is absent, the value of E_1 does not change. This constancy is of great interest when we consider how widely
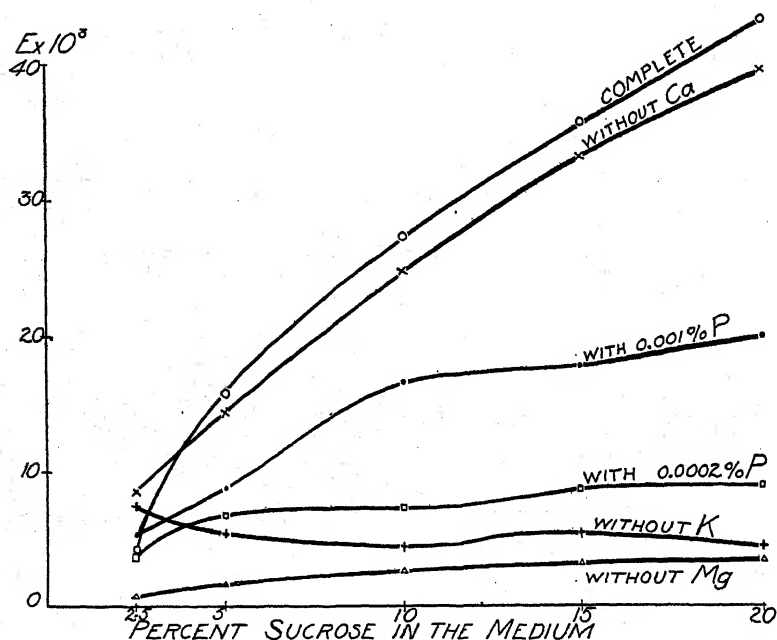
FIG. 1. The E_1 of *Penicillium glaucum* cultures of different salt supplements.

the values used in the calculation of E_1 vary. The work of Doby and Hibbard with sugar-beets (2), and that of Doby and Kertesz, and of Doby and Feher with *Penicillium* show that, if 1 of the important ions is lacking, the organism produces a much larger amount of saccharase per unit of tissue than when completely nourished.

As may be seen from Table II, the dry matter yield of the mold grown without potassium increases with increasing sucrose nour-

ishment from 0.13 to 0.42 gm., and the $If \times 10^{-3}$ changes from 57.2 to 10.1, but the value of E_1 is nearly constant. It is noteworthy that the value of E_1 generally is lower when an ion very important to growth, such as potassium or magnesium, is lacking from the medium, than if one of less importance, such as Ca, is lacking. The changes caused by variation in the quantities of phosphorus supplied are typical. Without phosphorus the mold does not grow at all. If 0.0002 per cent of P is present, there is some growth, and the E_1 is about that of the potassium- and magnesium-free cultures. After further increasing the P nourishment

TABLE III

Saccharase Content of Growing Penicillium Cultures

Complete salts, 5 per cent sucrose, on 50 cc. of medium, at 25°.

Age	Sugars in medium			Yield in dry matter	Saccharase in mold			Used for determination	Saccharase in medium		Saccharase in		
	Sucrose	Invert sugar	Total sugars		$k \times 10^4$	Dry matter at hydrolysis	$If \times 10^3$		Sucrose in whole reaction mixture	$k \times 10^4$	Mold, $E_1 \times 10^3$	Medium, $E_2 \times 10^3$	Total, $E_1 + E_2 \times 10^3$
days	per cent	per cent	per cent	gm.		gm.		cc.	gm.				
2	3.80	0.61	4.41	0.057	1.89	0.0406	13.9	30	4.14	0.1	0.80	0.07	0.87
4	1.59	2.35	3.94	0.396	20.74	0.1746	35.6	30	3.48	13.28	14.10	7.71	21.81
7	1.56	2.04	3.60	0.662	12.99	0.4340	9.0	30	3.47	3.33	5.94	1.93	7.87
11	1.34	1.71	3.05	0.857	3.88	0.3187	3.7	35	3.47	1.02	3.17	0.51	3.68
16	0.70	1.20	1.90	1.014	2.88	0.3000	2.8	30	3.21	1.54	2.84	0.82	3.66

the curve of the E_1 approaches to the E_1 curve of the fully nourished mold cultures.

Saccharase Content of Growing Penicillium Cultures

The distribution of saccharase between the mycelium and the medium will be discussed in a following paper. It will merely be shown here that relatively much less saccharase is found in the medium than in the mycelium. The data are given in Table III.

The amount of saccharase begins to decrease as soon as the greater part of the sucrose has been hydrolyzed. This fact is in

agreement with the result of some earlier investigations on the relation between the sugar content and enzymes produced by mold cultures (9, 10). But it was possible to show the presence of a very small amount of saccharase by a qualitative test (11), even after 3 weeks.

SUMMARY

1. The total enzyme content of the mycelium of a mold culture may be expressed by the formula

$$E_1 = \frac{k \times \text{gm. of sucrose}}{\text{dry matter}} \times (\text{total dry matter yield of the culture in gm.})$$

in which k is the monomolecular reaction constant, sucrose is the total sucrose content of the reaction mixture in gm., and dry matter the amount of dry mycelium in gm. which supplied the enzyme used.

2. In the same way the total saccharase content of the medium can also be expressed. But in this case the calculation is made on the basis of volume rather than weights.

$$E_2 = \frac{k \times \text{gm. of sucrose}}{\text{cc. of medium used}} \times (\text{volume of entire culture medium in cc.})$$

3. By adding these two values ($E_1 + E_2$) a number is obtained which indicates the saccharase, or other enzyme, yield of the whole culture.

4. In duplicate cultures growing on identical media on the 5th day the E_1 is practically the same, in spite of the fact that the enzyme activity (If_1) and dry matter yield vary greatly.

5. With complete salt nourishment the E_1 increases as the sucrose content of the medium is increased. In the absence of one important nutrient ion (K, P, Mg) and increasing sucrose supply, the value of E_1 is roughly constant.

6. In *Penicillium glaucum* cultures growing normally, the value of $E_1 + E_2$ reaches a maximum in the 1st few days; afterwards it decreases rapidly.

7. The use of the E values makes it possible to study the saccharase yield of mold cultures, especially the study of the distribution of the enzyme between mycelium and medium, since the values obtained are directly comparable.

8. The proposed method is applicable to the estimation of other enzymes in fungal or bacterial cultures, since the same principles apply to the determinations of other enzymes as well.

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A STUDY OF GLUTATHIONE

V. THE SPONTANEOUS CLEAVAGE OF GLUTATHIONE IN AQUEOUS SOLUTION

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In 1929, Hopkins (4) showed that when glutathione was boiled with water considerable decomposition occurred and that among the products were pyrrolidonecarboxylic acid and cysteinyl-glycine anhydride. In a previous publication (6) it was reported that glutathione splits into pyrrolidonecarboxylic acid and cysteinyl-glycine when it is incubated in water solution at 37°. It has also been shown that this cleavage is complete in 120 hours at 62° whereas only a small amount of cysteinyl-glycine anhydride is formed (7). At 37° none of the anhydride is formed. At 62° the extensive decomposition observed by Hopkins at the boiling temperature does not occur. In this paper these observations will be extended and their relationship will be shown to the ereptic digestion of glutathione which was previously reported.

It has been found that pyrrolidonecarboxylic acid is a primary product of the spontaneous cleavage of glutathione. Glutamic acid does not change to the pyrrolidonecarboxylic acid when incubated at 37° in solution alone or in a solution of glutathione. This fact precludes the possibility that glutamic acid is first eliminated from the molecule by hydrolysis and is then converted into pyrrolidonecarboxylic acid. This possibility could not be neglected since Foreman (2) has shown that a few hours boiling with water will bring about as much as 50 per cent conversion of glutamic acid into the pyrrolidonecarboxylic acid. The same result might have been accomplished in 2 weeks at 37°. However, the evidence shows that the formation of the pyrrolidone ring must occur before the peptide link is broken.

The spontaneous formation of pyrrolidonecarboxylic acid from

glutathione at physiologic temperatures suggests that this may be the first step in the synthesis of proline and hydroxyproline.

The identity of the other fragment of glutathione as a dipeptide of cysteine and glycine is established by the fact that two-thirds of the nitrogen of the glutathione used is precipitated with mercuric sulfate and by the identification of cysteine and glycine as the products of hydrolysis of the material thus precipitated. That it is cysteinyl-glycine is shown by the location of the free amino group on the cysteine residue (7). The material which was isolated from the mercury precipitate was not the pure dipeptide, however. The nitrogen content of various preparations was invariably low; it was about 80 per cent of that calculated for cysteinyl-glycine. The nature of the impurities is unknown. Cysteine was not present since cystine could not be separated even in traces after a solution of the preparation had been aerated at neutral reaction. Addition of cuprous oxide to the material dissolved in 0.5 N sulfuric acid did not yield an insoluble cuprous salt.

The response of cysteinyl-glycine to Sullivan's test for cysteine is of considerable interest. The material isolated gave a purplish red color which was quite distinct from the color given by cysteine. The difference in color was readily revealed in the colorimeter. The two colors were so divergent in shade that they could not be matched. However, when the color was faint it was practically impossible to distinguish between cysteine and cysteinyl-glycine, particularly when there was a relatively large amount of glutathione present. A small amount of cysteine (0.5 mg.) with cysteinyl-glycine (3 mg.) gave a color which was indistinguishable from that given by cysteine alone although it was more intense than the color produced by the amount of cysteine present. It required 5 mg. of the preparation used to produce a color of approximately the same intensity as 1 mg. of cysteine. The purple tint was not due to the presence of iron since it was not changed by addition of 2 cc. of a 5 per cent solution of sodium cyanide. Johnson and Voegtlin (5) noted a purple color when some of their early preparations of glutathione were subjected to Sullivan's test. It seems probable that these preparations were contaminated with cysteinyl-glycine.

On the basis of a positive Sullivan's test Meldrum and Dixon (8) concluded that certain preparations of crystalline glutathione

contained cysteine and that a solution of glutathione which had been incubated for 12 hours at 40° also contained cysteine. In view of the ease with which the glutamyl radical is lost and the fact that cystine could not be isolated even after 2 weeks of incubation it is probable that the test which they called positive was due to cysteinyl-glycine. Their incubation experiment was repeated at 37° for 19 hours. A very definite color was produced by an amount of solution corresponding to 1 mg. of glutathione but it was distinctly not the color given by cysteine either alone or in the presence of glutathione. It was the same color as that given by the cysteinyl-glycine preparation. An amount corresponding to 5 mg. of glutathione gave about as much color as 0.3 mg. of cysteinylglycine, indicating a decomposition of 5 or 6 per cent.

The tests just described were carried out according to Sullivan's (11) latest procedure (1929) while apparently Meldrum and Dixon used one of the two procedures described in 1926 (10). These early procedures give much less color with both cysteine and cysteinyl-glycine, and with Sullivan's test as first described it is practically impossible to distinguish between the two substances when dealing with small quantities. It was necessary to use at least 5 mg. of the cysteinyl-glycine preparation in order to obtain enough color to distinguish it from cysteine. The 1929 procedure gives enough color for this purpose with less than 1 mg. When the early procedures were used 5 mg. of the glutathione that had been incubated overnight at 37° gave a test that could be called positive with certainty only when compared with a blank prepared from a fresh solution of glutathione. The color was too faint to make it possible to judge whether the tint was different from the color given by a very small quantity of cysteine.

Meldrum and Dixon believe that their evidence tends to show that cysteine is one of the catalytic factors involved in the autooxidation of glutathione but stated that their quantitative experiments indicate that the active substance is somewhat more active than cysteine. Although one cannot say positively that traces of cysteine are entirely absent from preparations of glutathione, the evidence just given makes it highly improbable that significant amounts of cysteine can be present. On the other hand, a trace of cysteinyl-glycine may easily be present if the glutathione has stood for any length of time in water solution during the prepara-

tion.   This substance, in contrast to glycyl-cysteine which was found
not to have catalytic properties, has a free amino group in the
same relation to the sulfhydryl group as in cysteine.   It is probable
that this particular structure would allow it to enter into complexes
with metals just as cysteine does.   It is suggested that cysteinyl-
glycine is probably the factor that is present in preparations of
glutathione which with metals catalyzes the autoxidation of the
glutathione.

It should be emphasized that cysteinyl-glycine is very likely
to be a contaminant of glutathione preparations.   It is extremely
unlikely that glutamyl-cysteine or cysteine would be present.
It would be necessary for these substances to be present in the
tissues from which the glutathione is isolated or to be produced by
enzyme action during the process of extraction since the evidence
shows that in subsequent operations only pyrrolidonecarboxylic
acid and cysteinyl-glycine could be formed from glutathione.   The
evidence submitted by Hopkins and his coworkers to show that
glutathione was glutamyl-cysteine has perhaps given rise to the
idea that glycine is easily split off and that glutamyl-cysteine was
present in the early preparations.   The present evidence shows
that the link between cysteine and glycine is very stable.   There
is no evidence to show that glutamyl-cysteine is present in gluta-
thione preparations isolated from yeast and the evidence for the
presence of cysteine is very doubtful.

Brand, Harris, and Biloon (1) have shown that cystine dimethyl
ester, after reduction with cyanide, gives a positive Sullivan's test
while I have found that cysteine ethyl ester gives the same color
as cysteine in this test (7).   Cysteinyl-glycine is a third derivative
of cysteine in which the cysteine carboxyl is in combination, yet,
which gives a red color when subjected to Sullivan's test.

It was previously reported that erepsin hydrolyzes glutathione
into its constituent amino acids very slowly (6).   The slowness of
the action was found to be due partly to the use of too great a
concentration of substrate.   When the concentration of gluta-
thione was reduced to 0.05 M the action was complete in 4 to 5
days.   Since this is remarkably slow for the action of erepsin,
the hydrolysis was followed by titration with alkali in methyl
alcohol according to the method of Waldschmidt-Leitz and Har-
teneck (12).   It was thus found that only one carboxyl group is

liberated although both peptide links are broken. This result is interpreted to mean that erepsin cannot attack the glutathione molecule at the link between the glutamic acid and cysteine. This is to be expected since the free amino group is in the γ position. However, when the glutamyl radical has been split off as pyrrolidonecarboxylic acid the resulting cysteinyl-glycine is easily attacked by the erepsin. Thus the entire molecule is broken up with the appearance of only one carboxyl group. It is also apparent why the previous attempts to isolate glutamic acid from the hydrolysis mixture were unsuccessful.

Grassmann, Dyckerhoff, and Eibeler (3) have described the hydrolysis of oxidized glutathione into glycine and diglutamylcystine by means of the carboxypolypeptidase of the pancreas. No evidence of such an action on reduced glutathione has been observed with the crude glycerol extracts of intestinal mucosa which were used in this work.

Experimental Data

Pyrrolidonecarboxylic Acid from Glutathione Incubated at 62°— Three 3 gm. samples of crystalline glutathione were incubated at 62° for 163 hours in order to obtain cysteinyl-glycine (7). The filtrates from the mercury precipitates were treated as previously described (6) and finally evaporated to dryness. The residues were extracted with hot alcohol and ethyl acetate. The solvents were evaporated and the solids dissolved as much as possible in boiling ethyl acetate. The filtered solutions were evaporated to small volumes and the pyrrolidonecarboxylic acid was allowed to crystallize. From the three samples were obtained 0.70, 0.80, and 0.86 gm. of the acid which melted at 155°, 155°, and 158°, respectively. These amounts are 57, 65, and 70 per cent of theory. The material which was insoluble in alcohol and ethyl acetate amounted to 0.12, 0.07, and 0.23 gm., respectively. The last was dissolved in 0.8 ml. of water and the solution was saturated with hydrogen chloride at 0°. There was no precipitate after the solution had stood in the ice box for 2 weeks. Obviously not more than a trace of glutamic acid is formed when glutathione is incubated at 62°.

Incubation of Glutamic Acid with Glutathione—As a preliminary experiment a solution of glutamic acid was kept at 37° for 14 days.

From 1.0 gm. of glutamic acid used 0.94 gm. was recovered. Alcohol-soluble material was not present.

3 gm. of glutathione and 0.5 gm. of glutamic acid dissolved in about 50 cc. of water were kept at 37° for 14 days.   A solution of mercuric sulfate in sulfuric acid was then added to precipitate the sulfur-containing substances.   The precipitate was separated and the excess mercury removed from the solution with hydrogen sulfide.   The solution then contained 143 mg. of nitrogen.   After removal of the sulfuric acid with barium hydroxide, it was evaporated at a low temperature under reduced pressure.   The residue was extracted with hot alcohol.   The extract was filtered and evaporated to dryness.   This residue was treated with boiling ethyl acetate which left a small amount undissolved.   On evaporation of the ethyl acetate there was obtained 0.59 gm. of pyrrolidonecarboxylic acid which melted at 158° (uncorrected).

The material which was insoluble in alcohol and ethyl acetate weighed 0.45 gm. and melted with decomposition at 195° (uncorrected).   It had the properties of glutamic acid but was probably contaminated with a small amount of impurities since there is always a small residue insoluble in alcohol and ethyl acetate.

As a control 3.0 gm. of glutathione were treated as just described.   A summary follows.

	Glutathione plus 0.5 gm. glutamic acid	Glutathione alone
	gm.	*gm.*
Nitrogen in filtrate from mercury precipitate...	0.143	0.101
Residue insoluble in ethyl acetate...............	0.45	0.07
Pyrrolidonecarboxylic acid.....................	0.59	0.62

The difference between the nitrogen contents of the two filtrates (0.042 gm.) is equivalent to 0.44 gm. of glutamic acid.   The difference between the two insoluble residues, which is the amount of glutamic acid recovered, is 0.38 gm.   The recovery of glutamic acid was therefore 86 per cent of the amount present in the filtrate after removal of the mercury with hydrogen sulfide.   Practically identical amounts of pyrrolidonecarboxylic acid were isolated. The small insoluble residue which is normally found in the filtrate from the mercury precipitate does not have the properties of glutamic acid, as shown in the previous section.

Cysteinyl-Glycine—The separation of this substance has already been described (7). The nitrogen contents of three samples are given below.

	N by Kjeldahl *per cent*
Sample 1	12.65
" 2	12.58
" 3	13.50
Calculated for $C_5H_{10}O_3N_2S$	15.73

Digestion of Glutathione by Erepsin—A solution containing 1.54 gm. of crystalline glutathione and 15 ml. of a glycerol extract of intestinal mucosa in 100 ml. of m/15 phosphate buffer adjusted to pH 7.8 was incubated at 37°. At intervals 10 ml. samples were withdrawn and titrated with 0.2 N alcoholic potassium hydroxide according to the procedure of Waldschmidt-Leitz and Harteneck (12). In 5 days the titration reached a constant value which was 2.03 ml. of 0.2 N alkali greater than the initial titration. This is 81 per cent of the amount of alkali required to neutralize one carboxyl group. Cystine was isolated from 10 ml. after aeration. The yield was 47 mg. or 79 per cent of theory. Since it has been shown that glutathione is γ-glutamyl-cysteinyl-glycine (6, 7, 9), cysteine can appear only when the molecule has been completely hydrolyzed. Obviously, the molecule was completely broken up with the liberation of only one carboxyl group.

Toward the end of the experiment a difficulty was encountered because of the separation of some cystine. It was thought possible, however, to obtain a fairly uniform sample by sampling quickly after shaking the mixture thoroughly. The cystine was dissolved by addition of a known amount of normal sulfuric acid and the amount of alkali necessary to neutralize this added acid was then subtracted from the total amount used. It may be that this procedure led to a somewhat low result inasmuch as a proportional amount of cystine may not have been withdrawn with the solution. However, this error could not be serious and the result is adequate to show that only one carboxyl group is liberated.

SUMMARY

When glutathione is kept in aqueous solution at 37–62° it undergoes cleavage into pyrrolidonecarboxylic acid and cysteinyl-

glycine. The pyrrolidonecarboxylic acid is a primary product of this cleavage. It is suggested that this formation of pyrrolidonecarboxylic acid may well be the first step in the synthesis of proline.

With Sullivan's test for cysteine, cysteinyl-glycine gives a purplish red color which is distinct from that given by cysteine. If the color is faint, however, it cannot be distinguished from the color due to a very small quantity of cysteine. It is suggested that this behavior of cysteinyl-glycine has led Meldrum and Dixon to attribute to cysteine a catalytic activity in the autooxidation of glutathione which is probably due to cysteinyl-glycine.

Erepsin does not attack glutathione directly but hydrolyzes the cysteinyl-glycine when the glutamyl radical has been broken off as pyrrolidonecarboxylic acid. The glutathione molecule is in this way completely broken up with the liberation of only one carboxyl group.

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THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI

XXII. CONCERNING THE CARBOHYDRATES OF THE PURIFIED WAX*

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INTRODUCTION

In a previous paper (1) we have described the ether-soluble constituents which are obtained on saponifying the purified wax from the human type of tubercle bacilli, Strain H-37, and at the same time a preliminary study was reported of the water-soluble fraction. It was found that the water-soluble material amounted to about 40 per cent and that it contained an organic phosphoric acid together with reducing sugars which gave pentose color reactions. From the aqueous solution two osazones were isolated. The more soluble osazone melted at 165–166° while the less soluble melted at 210°. The osazone which melted at 165–166° gave a depression of the melting point when mixed with *l*-arabinosazone and the opinion was therefore expressed that the pentose was not arabinose.

During the past year we have continued the investigation of the water-soluble constituents which are obtained from the purified wax and we have been able to isolate and identify several sugars which are present in this mixture. By means of diphenyl- and benzylphenylhydrazines we have separated from the aqueous solu-

tion the hydrazones of *d*-arabinose and *d*-galactose.   In addition to these sugars, traces of mannose, inosite, and glucosamine have been isolated and identified.

The occurrence of *d*-arabinose among the cleavage products of the so called tubercle bacilli wax is of some interest because this pentose is very seldom found in nature.   It was shown by Neuberg (2) that the pentose excreted in a case of pentosuria was *dl*-arabinose and the same author showed later that *d*-arabinose could be separated from the *dl* mixture by means of *l*-menthyl-hydrazine (3).   The only other instance of the presence of *d*-arabinose in a natural product that we have found mentioned in the literature was recorded some years ago by Léger (4) who found that barbaloin, on prolonged hydrolysis with alcoholic hydrochloric acid, yields *d*-arabinose and aloemodin.   The latter work has been confirmed by Gibson and Simonsen (5).

It was stated in the former paper (1) that saponification of the purified wax by alcoholic potassium hydroxide yielded unsaponifiable wax, fatty acids, and a large quantity of a gummy mass which was insoluble in alcohol but easily soluble in water.   The latter substance did not reduce Fehling's solution until it had been boiled for some time with dilute acid.

The reaction products obtained on alkaline saponification have been further investigated and we have found that the water-soluble component represents a complex polysaccharide which on hydrolysis with dilute acid yields reducing sugars.   It has been impossible so far to identify all of the sugars which are liberated on hydrolysis but a small amount of mannose and larger amounts of *d*-arabinose and *d*-galactose have been isolated.

In connection with the saponification or hydrolysis of the purified wax it is of interest to notice that boiling alcoholic potassium hydroxide removes only the ether-soluble constituents, namely unsaponifiable wax and fatty acids, while the polysaccharide portion of the molecule is apparently left intact.   On the other hand, boiling alcoholic hydrochloric acid not only liberates the ether-soluble constitutents but causes also a partial hydrolysis of the polysaccharide as shown by the formation of reducing sugars.

The work is far from complete.   It has been impossible to account for but little more than one-half of the total sugars which are formed when the wax is hydrolyzed.   A complex organic phos-

phoric acid is also present which has not been identified.   We have only been able to isolate a trace of glucosamine, thus leaving the bulk of the nitrogen compounds still unknown.

The material used in this investigation represented the water-soluble constituents obtained from 50 gm. of purified wax after this substance had been boiled with alcohol containing 2 per cent of hydrochloric acid.   The unsaponifiable wax and fatty acids had been extracted with ether and examined immediately as has already been described (1).   The aqueous alcoholic solution containing the water-soluble material had stood in a stoppered flask for about 18 months before the present examination could be started.

The straw-colored solution which measured about 800 cc. was concentrated to dryness under reduced pressure.   The residue was freed as far as possible of hydrochloric acid by repeated evaporation *in vacuo* after adding water and alcohol.   The dark brown syrup which was obtained was dissolved in 150 cc. of water and the solution was made faintly alkaline with barium hydroxide.   The solution was mixed with 2 volumes of alcohol when a fine amorphous precipitate formed which settled slowly to a gummy mass on the bottom of the flask.   The supernatant solution was decanted and the precipitate was rinsed with alcohol.

The solution and washings were combined and reserved for the isolation of sugars.

Examination of Barium Precipitate

The crude barium salt was treated with a little cold water and the insoluble matter, consisting of barium phosphate, was filtered off, washed with water, and discarded.   The filtrate was mixed with alcohol when an amorphous barium salt was precipitated. The substance was reprecipitated several times in the same manner until a white amorphous powder was obtained.   The purified product weighed 2 gm.   The substance was easily soluble in water but the aqueous solution gave no precipitate with lead acetate.   It contained nitrogen and it gave a test for reducing sugar after acid hydrolysis.

For analysis the substance was dried at 105° *in vacuo* over dehydrite. The loss in weight was 5.09 per cent.

Found. N 3.73, P 5.06, Ba 27.88 per cent

By means of the Van Slyke method (6) it was found that about one-half of the nitrogen was present as amino nitrogen.

On hydrolysis with 5 per cent sulfuric acid the reducing sugar, determined by the Shaffer-Hartmann method (7) and calculated as glucose, attained a constant value of 13 per cent after the solution had been boiled for 2 hours. We were unfortunately unable to identify any of the cleavage products.

It was stated in the former paper (1) on the analysis of the purified wax that, "Practically all of the phosphorus was present in organic combination, presumably as glycerophosphoric acid." In view of the results mentioned above it is evident that the organic phosphoric acid is not glycerophosphoric acid but a much more complex compound.

Examination of Filtrate Containing the Sugars

The filtrate from the barium salt was freed quantitatively of barium with sulfuric acid. The barium sulfate was removed and the solution was concentrated under reduced pressure. The mixture contained reducing sugar and nitrogen but it was found that the amount of reducing sugar was greatly increased when the solution was boiled with dilute acid. An attempt to separate the simple from the complex sugars by treatment with various solvents was unsuccessful and since it was impossible to isolate any crystalline substance the whole solution was evaporated to dryness *in vacuo*.

A portion of the dried syrup was hydrolyzed with 5 per cent sulfuric acid. It was found that a maximum of reducing sugar amounting to 93 per cent, calculated as glucose, was obtained after the solution had been boiled for 1 hour. After the sulfuric acid had been removed quantitatively with barium hydroxide the solution was concentrated *in vacuo* but it was impossible to isolate any crystalline substance from the hydrolysate. Osazones were therefore prepared in the usual manner by heating the solution with phenylhydrazine hydrochloride and sodium acetate. The crude osazone, which was partly non-crystalline, was fractionated by

treatment with acetone, alcohol, and benzene yielding a low melting and a high melting osazone in the form of yellow needle-shaped crystals. The low melting product resembled arabinosazone and it melted at 164°. The presence of pentose sugars in the hydrolysate was further confirmed by means of color reactions. The high melting fraction resembled glucosazone and it melted at 207°. These osazone fractions were similar to those described in the former paper (1).

Determination of Pentose Sugars

Since the solution contained some pentose, possibly arabinose, in addition to other sugars some quantitative determinations were made of pentose sugars by the usual furfural distillation method (8). The amount of phloroglucide obtained corresponded to 28, 32.5, and 33 per cent of pentose.

Isolation of Glucosamine Hydrochloride and Inosite

The residual solutions from the furfural distillations were combined, decolorized with norit, and evaporated under reduced pressure to dryness. A small amount of syrup remained which gave a reaction for reducing sugar and when it was boiled with sodium hydroxide ammonia was liberated. When the syrup was allowed to stand some crystalline material, consisting mostly of inorganic chlorides, separated. The material was treated with cold concentrated hydrochloric acid and the insoluble inorganic chlorides were filtered off. The solution was evaporated and the treatment with hydrochloric acid was repeated several times. Finally a few large colorless rhombic crystals separated when the solution was slowly concentrated. The crystals, after they had been collected and washed with a little ice-cold hydrochloric acid, were identified as glucosamine hydrochloride by means of their crystallographic properties.

The filtrate from the glucosamine hydrochloride was slowly evaporated in a desiccator when a few prismatic or needle-shaped crystals were obtained. The optical properties of these crystals were identical with those of inosite and the identification was further confirmed by the reaction of Scherer.

The amount of glucosamine hydrochloride and inosite obtained, as mentioned above, was very small. In order to secure more of

these substances the following experiments were carried out.   (a)
10 gm. of the purified wax were refluxed for 3 hours with 40 cc. of
concentrated hydrochloric acid and 30 cc. of water.   The material
decomposed leaving a black mass in the flask.   The insoluble
matter was filtered off; the solution was decolorized with norit and
concentrated to a thick syrup.   The latter was treated with ab-
solute alcohol and the insoluble portion was dissolved in a little
dilute hydrochloric acid.   The solution was concentrated slowly
in a desiccator when a small amount of colorless rhombic crystals
was obtained which appeared to be identical with glucosamine
hydrochloride.   (b) In this experiment 10 gm. of the purified
wax were hydrolyzed by boiling with alcohol containing 2 per cent
of hydrochloric acid.   The mixture was diluted with water and the
unsaponifiable wax and the fatty acids were extracted with ether.
The aqueous solution was evaporated to dryness and the residue
was boiled with 20 per cent hydrochloric acid until the pentoses
and other sugars were decomposed.   The carbonaceous material
was filtered off and the filtrate, after it had been decolorized with
norit, was evaporated to a syrup.   The syrup was treated exactly
as described above under (a) and a small amount of glucosamine
hydrochloride was obtained in the form of colorless rhombic crys-
tals.   The two lots of crude glucosamine hydrochloride were com-
bined, weight about 50 mg., and recrystallized from dilute hydro-
chloric acid.   The colorless rhombic crystals that separated
weighed about 10 mg.   The optical properties of these crystals
were compared with those of pure glucosamine hydrochloride which
had been recrystallized from dilute hydrochloric acid and they
were found to be identical.

Optical Properties of Glucosamine Hydrochloride[1]

Glucosamine hydrochloride crystals present rhombic, sphenoidal,
or hexagonal outlines.   The crystals are biaxial negative with 2V
$\doteqdot$ 45°, and they have a very low birefringence. $\beta \doteqdot 1.563$.   The
acute angle of the rhombic and hexagonal forms and one angle of
the sphenoidal form is 75°.   This angle is always bisected by γ.   It
is somewhat difficult to obtain good interference figures.

[1] We are indebted to Dr. E. J. Roberts of this Laboratory for making
these measurements.

The final mother liquors from (*a*) and (*b*) were combined and slowly concentrated in a desiccator when a small amount of needle-shaped crystals was obtained. The crystals were isolated and recrystallized from a little water by adding alcohol. The colorless prismatic needles that separated weighed about 10 mg. They were identical in form with inactive inosite and gave the Scherer reaction.

It is evident from these results that the purified wax contains small quantities of glucosamine and inosite. The amount of glucosamine that can be isolated is so small, however, that it represents only a minor fraction of the nitrogen contained in the wax.

Isolation of d-Arabinose Diphenylhydrazone

The presence of a pentose in the sugar mixture obtained on hydrolyzing the water-soluble syrup was indicated by color reactions, osazone, and by the furfural formation on distillation with hydrochloric acid. In the first isolation of a pentose derivative we employed diphenylhydrazine and obtained a good yield of hydrazone by the following method. The dried water-soluble syrup, 2 gm., was hydrolyzed by boiling 5 per cent sulfuric acid. The sulfuric acid was removed quantitatively with barium hydroxide and the solution was concentrated *in vacuo* to a volume of 20 cc. To this solution was added 1 gm. of diphenylhydrazine hydrochloride, 1.5 gm. of sodium acetate, and 30 cc. of alcohol and the mixture was refluxed for 30 minutes. Colorless needles began to separate and after the mixture had stood overnight the crystals were filtered off and washed with 50 per cent alcohol and with ether. The dried substance weighed 0.8 gm. and after it had been recrystallized from 60 per cent alcohol 0.64 gm. of beautiful colorless needles was obtained.

Analysis—0.1604 gm. substance: 12.6 cc. N_2 at 20° and 763 mm.
$C_{17}H_{20}O_4N_2$ (316). Calculated. N 8.86 per cent
Found.   " 9.15 "   "

That the substance was *d*-arabinose diphenylhydrazone was shown by the properties mentioned below. When heated at the rate of a little more than 1° per second the hydrazone melted at 210°; *l*-arabinose diphenylhydrazone melted at the same temperature but a mixture of the two hydrazones melted at 198°. When

heated at the rate of 1° per second the hydrazone melted at 208°; *d*-arabinose diphenylhydrazone melted at the same temperature and there was no depression of the melting point of a mixture of the two products.

The rotation of 0.1 gm. of the hydrazone in 4 cc. of pyridine plus 6 cc. of absolute alcohol in a 1 dm. tube was −0.17°.   The rotation of *d*-arabinose diphenylhydrazone under the same conditions and concentration was −0.17°, while *l*-arabinose diphenylhydrazone gave under similar conditions a reading of +0.24°.

Isolation of d-Arabinose Benzylphenylhydrazone

We were anxious to secure a sufficient quantity of the *d*-arabinose hydrazone to permit us to prepare some of the free pentose from it.   Since diphenylhydrazine was not available we employed benzylphenylhydrazine.

The remainder of the dried syrup, 8.8 gm., was hydrolyzed with 5 per cent sulfuric acid after which the acid was removed in the usual manner and the solution was then mixed with 75 cc. of alcohol and 5 gm. of freshly distilled benzylphenylhydrazine. The mixture was warmed until a clear solution resulted.   The hydrazone began to crystallize after a few minutes.   After standing for 3 hours at room temperature and 2 hours in ice water, the crystals were filtered off and washed with 75 per cent alcohol and with ether.   The yield of dry material was 3.43 gm.   The substance was twice recrystallized from 75 cc. of 75 per cent alcohol and we recovered 2.7 gm. of fine colorless needles.   Heated in a capillary tube the hydrazone melted at 174–175° and there was no depression of the melting point when the substance was mixed with pure *d*-arabinose benzylphenylhydrazone.

The filtrate from the *d*-arabinose benzylphenylhydrazone was examined for other sugars but, except for a trace of inosite, it was impossible to separate any definite crystalline substance from this mixture.

Preparation of d-Arabinose

The hydrazone, 2.4 gm., was decomposed by refluxing it for 5 hours with 45 cc. of 75 per cent alcohol containing 3 gm. of benzaldehyde.   After the mixture had stood overnight the precipitated benzaldehyde hydrazone was filtered off and washed with water. The filtrate and washings were combined, extracted ten times with

ether, and then evaporated under reduced pressure to dryness. The residue which formed a crystalline mass was dissolved in water, treated with norit, and concentrated in a vacuum desiccator. The crystals were stirred up with alcohol, filtered, and washed with alcohol. After the substance had been recrystallized twice in the same manner 0.7 gm. of colorless dense needle-shaped crystals was obtained. The crystals melted at 160–161° and there was no depression of the melting point when the substance was mixed with a sample of Eastman d-arabinose. Rotation: 0.4 gm. of the crystals was dissolved in water and made up to 10 cc. The solution showed mutarotation and the following readings were made in a 1 dm. tube. 10 minutes after the solution had been prepared the rotation was −5.03° but after 40 minutes the constant value of −4.18° was attained, hence $[\alpha]_D^{25} = -104.5°$.

Preparation of the Polysaccharide from the Purified Wax

The purified wax, 10 gm., was refluxed for 6 hours with 200 cc. of 2.5 per cent alcoholic potassium hydroxide. During the boiling the odor of ammonia was distinctly noticeable at the top of the condenser. The wax disintegrated gradually with the formation of a colorless oil consisting of unsaponifiable wax, and an alcohol-insoluble amorphous mass remained on the bottom of the flask while the alcoholic solution contained the soaps of the fatty acids. The warm solution which contained the unsaponifiable wax in suspension was decanted and the insoluble residue was rinsed with hot alcohol and with chloroform. The residue was then transferred to a small Buchner funnel and washed several times with warm chloroform in order to remove adhering unsaponifiable wax. After the material had been dried it was dissolved in 60 cc. of water; the solution was acidified with acetic acid and the fine suspension of waxy material was removed by filtering through a layer of norit on a small Buchner funnel. The filtrate was mixed with alcohol when a precipitate formed which gradually settled out as a gummy mass. The supernatant liquid was decanted and the insoluble precipitate after it had been washed with alcohol was dissolved in dilute acetic acid and again precipitated with alcohol. After repeating this treatment the material was dissolved in about 20 cc. of water and the solution was poured slowly with constant stirring into 300 cc. of absolute alcohol. A voluminous amorphous precipitate was

obtained which was filtered off, washed with alcohol, and dried *in vacuo*. The substance was a non-hydroscopic nearly white powder and it weighed 3.1 gm.

Hydrolysis of the Polysaccharide

Isolation of Mannose Phenylhydrazone

The crude polysaccharide, 2 gm., was hydrolyzed with 5 per cent sulfuric acid. The acid was removed and the solution was concentrated in the usual manner to a volume of 15 cc. The solution was treated with phenylhydrazine when 0.064 gm. of hydrazone separated. When the substance was recrystallized from 60 per cent alcohol typical mannose phenylhydrazone crystals were obtained. The yield of the crude hydrazone corresponds to 2.1 per cent of mannose.

Isolation of d-Arabinose Benzylphenylhydrazone

The excess of phenylhydrazine was removed by means of benzaldehyde and the solution was concentrated to 4 cc. The syrup was dissolved in 15 cc. of alcohol and 2 gm. of benzylphenylhydrazine were added. After the solution had been warmed slightly and allowed to stand a short time fine colorless needles began to separate. The crystals were filtered off after the mixture had stood for several hours and washed with alcohol and ether. The filtrate was allowed to stand for 48 hours and treated as mentioned below.

The crude hydrazone after it had been dried *in vacuo* weighed 1.58 gm. It was recrystallized from 75 per cent alcohol yielding 1.32 gm. of fine colorless needles. The substance melted at 174° and there was no depression of the melting point when mixed with *d*-arabinose benzylphenylhydrazone.

Calculated from the yield of the crude hydrazone the polysaccharide contained 35.9 per cent of *d*-arabinose.

Isolation of d-Galactose Benzylphenylhydrazone

The filtrate from the *d*-arabinose benzylphenylhydrazone was concentrated *in vacuo* and as the alcohol was removed a copious crystalline precipitate separated. The crystals were filtered off, washed with water, and dried *in vacuo*. The substance which

weighed 0.7 gm. was treated with cold acetone and recrystallized from 75 per cent acetone when 0.2 gm. of colorless needles was obtained. The hydrazone melted at 157° and there was no depression of the melting point when mixed with a sample of purified d-galactose benzylphenylhydrazone. The specific rotation in pyridine solution was −11.8°. The amount of crude hydrazone that was obtained corresponds to 17.5 per cent of galactose in the polysaccharide.

It was not possible to isolate any other crystalline substance from the mother liquor after the galactose benzylphenylhydrazone had been removed, although reducing sugars were still present. The amount of mannose, d-arabinose, and galactose isolated in the form of hydrazones represents only 55.5 per cent of the polysaccharide.

SUMMARY

1. A partial examination has been made of the sugars which are liberated when the purified wax from tubercle bacilli is hydrolyzed with dilute acid. It has been possible to isolate from the hydrolysate pure crystalline d-arabinose together with traces of glucosamine hydrochloride and inosite.

2. When the purified wax is saponified with alcoholic potassium hydroxide the ether-soluble constituents are liberated while a complex carbohydrate or polysaccharide remains as an alcohol-insoluble amorphous powder. The polysaccharide on acid hydrolysis yields mannose, d-arabinose, and galactose together with other as yet unidentified reducing sugars.

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THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI

XXIII. SEPARATION OF THE LIPOID FRACTIONS FROM THE TIMOTHY BACILLUS

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INTRODUCTION

The present report forms a part of a systematic investigation on tuberculosis which is conducted under the auspices of the Research Committee of the National Tuberculosis Association in cooperation with several research institutions. Previous work in this Laboratory on this program has concerned itself with the chemical composition of definite strains of the human (1), avian (2), and bovine (3) types of tubercle bacilli. It seemed desirable from many points of view to include in these comparative investigations of pathogenic bacteria at least one strain of a non-pathogenic acid-fast type and the timothy-grass bacillus was selected for this purpose.

The timothy-grass bacillus, *Mycobacterium phlei*, was isolated by Moeller in 1898 (4) from *Phleum pratense*. The organism grows in the form of rods which in appearance and staining properties resemble the tubercle bacilli and, in common with all acid-fast bacteria, it is characterized by a high lipid content. Published investigations on the timothy bacillus have dealt mainly with such subjects as the composition of the medium (5), the metabolism of the organism (6), or with the biological reactions which it produces (7). According to Damon (8) dried timothy bacilli contain a substance which stimulates growth in young rats

fed a ration free of vitamin B. A review of other properties and characteristics of non-pathogenic acid-fast bacteria including the timothy bacillus is given by Wells, DeWitt and, Long (9). Pfannenstiel (10) has published some data on the amount of lipids obtained when dried acid-fast bacteria are extracted with a mixture of acetone and ether. Long and Campbell (11) in a comparative study of the lipids of various acid-fast bacteria report that the timothy bacillus yielded 20.2 per cent of total lipid. The only work dealing exclusively with the chemical composition of the timothy bacillus was published by Coghill and Bird (12). These authors report that the dried bacilli gave 1.87 per cent of fat on extraction with ether; further extraction with chloroform yielded 0.85 per cent, but when the defatted bacteria were treated with hot 20 per cent hydrochloric acid and reextracted with chloroform an additional 14.93 per cent of lipid was obtained.

The timothy bacilli used in our experiments were grown on the Long (13) synthetic medium in the laboratories of Parke, Davis and Company. In the extraction of the bacteria and in the separation of the lipoid fractions we followed as closely as possible the procedure employed in this Laboratory in the investigation of the human tubercle bacilli (1).

Under similar conditions of procedure we have found that the timothy bacillus yields much less lipoid than the tubercle bacilli. The lipoids are, however, similar in character to those of the tubercle bacilli and consist of phosphatide, acetone-soluble fat, and wax; quantitatively the wax fraction predominates.

The timothy bacillus produces more pigment than the tubercle bacilli and the lipoid extracts are highly colored, ranging from deep orange to dark red, depending upon the concentration of the extract.

EXPERIMENTAL

The organism which was used in these experiments has been described as follows by Dr. William Charles White in a personal communication:

"*Mycobacterium phlei* (timothy-grass bacillus). H. K. Mulford No. 1589. Originally isolated by Alfred Moeller at Belzig Sanatorium prior to September 1904. Transferred through Dr. S. J. Maher to E. R. Baldwin, Saranac Lake, September 1904. Transferred on agar, two cultures, from E. R.

Baldwin to J. D. Aronson, Henry Phipps Institute, June 1923. This culture carried on glycerine agar and transplanted monthly by Dr. Aronson and culture sent to H. K. Mulford Company February 13, 1925. H. K. Mulford Company sent culture to Parke, Davis and Company February 16, 1928 and to Hygienic Laboratory, May 24, 1930."

At the Parke, Davis and Company laboratory the organism was known as culture No. 02145, and for the present work the cultures were grown in 1602 1 liter Pyrex bottles for a period of about 4 weeks. The bacteria were filtered off on June 27, 1928, washed with water, and placed in alcohol and ether, equal parts, contained in three 5 gallon Pyrex bottles and in one 2 gallon bottle. The solvent had been saturated with carbon dioxide. The material was transported to the Sterling Chemistry Laboratory and kept in a dark, cold room until January 25, 1930. The bottles were then brought into the laboratory and kept at room temperature for about 3 days with frequent shaking. The bacteria settled rapidly to a thick yellowish layer leaving a clear red-colored supernatant extract.

Throughout these experiments an atmosphere of carbon dioxide or nitrogen was used as far as possible and the solvents employed were saturated with these gases. All solvents were freshly distilled. The alcohol and ether had been distilled over potassium hydroxide.

The supernatant extract was siphoned off under carbon dioxide pressure and the bacterial residue was filtered on large Buchner funnels under a stream of carbon dioxide. After the bacteria had been washed with a mixture of alcohol and ether they were digested in chloroform as will be described later.

Examination of the Alcohol-Ether Extract

The ether was removed at a temperature of about 30° by means of a current of carbon dioxide and most of the alcohol was distilled off under reduced pressure. The residual solution which contained the lipoid material in suspension was shaken with ether yielding a dark reddish brown extract and the ethereal solution was washed with water. During the extractions and washings rather permanent emulsions formed. It was possible, as will be mentioned later, to separate from the aqueous emulsions a small quantity of a reddish brown pigment, Fraction T-7. The aqueous solution and washings were reserved for the isolation of the polysaccharide.

The washed ether extract was dried with sodium sulfate, filtered, and concentrated to a volume of about 4 liters.   The solution was filtered through a Chamberland candle under carbon dioxide pressure but there was no visible residue on the candle.   The ether was evaporated at a low temperature and the residue was dissolved in 400 cc. of ether when a small amount of an orange-colored insoluble substance separated which was filtered off and washed with ether.   This substance which was soluble in chloroform was combined with Fraction T-7.

Separation of the Phosphatide

The ethereal solution, after it had been concentrated to a volume of 300 cc., was mixed with 300 cc. of acetone when an oily material separated which soon collected as a brown mass on the bottom of the flask.   The solution was decanted and the residue was washed with cold acetone.   The washings were combined with the ether-acetone solution and the whole was allowed to stand for 24 hours.   The small amount of material that had separated was collected and combined with the main phosphatide fraction. For purification the crude phosphatide was precipitated ten times from 150 cc. or 100 cc. of ether by an equal volume of acetone. The material was finally dissolved in 80 cc. of ether and the solution was poured slowly with constant stirring into 350 cc. of ice-cold acetone.   The product separated as a yellow amorphous powder which was filtered off and washed first with acetone and finally with methyl alcohol.   After the substance had been dried in a vacuum desiccator it formed a yellow powder which weighed 18.7 gm.   This phosphatide is designated as Fraction T-1.   Heated in a capillary tube, it softened at 180°, turned dark in color, and melted at 190°.   The substance contained phosphorus and a trace of nitrogen but it was free from sulfur and halogen.

For analysis it was dried at 105° *in vacuo* over dehydrite.

0.4032 gm. substance: 0.0405 gm. $Mg_2P_2O_7$.
0.3332 "   "   : (Kjeldahl) 0.53 cc. 0.1 N HCl.
                    Found. P 2.80, N 0.22

Separation of the Acetone-Soluble Fat

The ether-acetone mother liquor from the phosphatide precipitation was placed in a freezing mixture of ice and salt when a slight

amount of sticky material separated on the sides of the flask. The solution was decanted and the flask was rinsed out with acetone. The ether-acetone solution was concentrated under reduced pressure to about one-third of its original volume, and it was again cooled. A slight amount of insoluble matter separated and was collected. The total amount of acetone-insoluble material weighed only 0.9 gm. and was probably impure phosphatide.

The acetone mother liquor was concentrated to dryness under reduced pressure and the fat residue was taken up in a little ether. The ether was distilled off and the residue was dried in a vacuum desiccator. The acetone-soluble fat which was obtained in this manner is designated Fraction T-2. It formed a dark reddish brown oily liquid which weighed 87.4 gm. The fat contained a trace of phosphorus but it was free from sulfur and nitrogen. The following constants were determined: saponification number 231.8, acid number 62.7, and iodine number 72.3.

Examination of the Aqueous Solution
Isolation of the Polysaccharide

The aqueous solution and washings mentioned above which remained after the lipoids had been extracted with ether measured about 20 liters. The solution was yellowish in color, not quite clear, and it showed an acid reaction on litmus paper. Owing to excessive foaming it could not be concentrated under reduced pressure. The solution was treated with an excess of lead acetate and the resulting precipitate was filtered off and washed with water. The filtrate was now concentrated *in vacuo* to a volume of about 1 liter and an excess of basic lead acetate was added. Hardly any precipitate formed until the solution was made alkaline with ammonium hydroxide when a voluminous amorphous lead salt separated. After standing for some time this precipitate was filtered off and washed with a little water. The filtrate and washings were concentrated *in vacuo* to 500 cc. and again treated with basic lead acetate and ammonia when a further precipitate was obtained. The precipitate was filtered off, washed with a little water, and combined with the first lot. The lead salt after it had been dried *in vacuo* was suspended in water and decomposed with hydrogen sulfide. After the lead sulfide had been removed the filtrate was concentrated *in vacuo* to a syrup and the latter was dried in a

vacuum desiccator.   The thick syrup was ground under absolute
alcohol in a mortar until a nearly white powder resulted and the
latter was filtered off, washed with absolute alcohol, and dried
in vacuo.   The filtrate was again concentrated to a thick syrup and
the latter was again ground under absolute alcohol as at first when
a smaller amount of a powder was obtained.   This was filtered off,
washed with absolute alcohol, dried, and combined with the first
lot.   The substance is apparently a polysaccharide and is desig-
nated as Fraction T-3.   It does not reduce Fehling's solution
directly but after it has been boiled with dilute acid for some time
it gives a heavy reduction with Fehling's solution.   The substance
was a nearly white non-hygroscopic powder which weighed 124 gm.

On combustion it left 1.96 per cent of ash.   It contains phos-
phorus and nitrogen but no sulfur.

Analysis
0.5361 gm. substance: (Kjeldahl) 9.95 cc. 0.1 N HCl.
0.6610 "   "   :   "   12.31 " 0.1 "   "
0.9099 "   "   : 0.0202 gm. $Mg_2P_2O_7$.
                  Found.   N 2.61, 2.62, P 0.62

Extraction of Bacterial Residue with Chloroform

The washed bacteria, after the alcohol-ether extraction, were
mixed with 4 liters of chloroform and allowed to stand at room
temperature with occasional stirring for 4 weeks.   The solvent
was then removed by filtration on large Buchner funnels and the
bacterial residue was again digested with 4 liters of chloroform.
The mixture was filtered and the bacteria were treated with alcohol
and ether as mentioned below.

The chloroformic extracts were combined and filtered through a
Chamberland candle under carbon dioxide pressure.   The filtrate
which was bright red in color was dried with sodium sulfate and
concentrated under reduced pressure to dryness.   The residue
formed a reddish wax-like mass and it weighed 146.6 gm.   This
substance represents the crude chloroform-soluble wax and it is
designated as Fraction T-4.

Extraction of Bacterial Residue with Alcohol and Ether

The bacteria, after the chloroform extraction, formed, on dry-
ing, a tough fibrous mass which it was very difficult to pulverize.

It was found, however, if the bacteria were treated with a mixture of alcohol and ether they formed, on drying, a friable mass which could be easily pulverized.   The total bacterial residue was therefore stirred up with 5 liters of equal parts of alcohol and ether. The solvent was filtered off on Buchner funnels and the residue was washed with alcohol-ether solution.   The filtrate was put through a Chamberland candle under carbon dioxide pressure, dried with sodium sulfate, and concentrated to dryness *in vacuo*. The residue was a yellowish wax-like mass which weighed 11.8 gm., and which is designated as Fraction T-5.

Extracted Bacterial Cells

The extracted bacteria which were of a yellowish brown color were dried in a vacuum oven at 40°.   The dried cell residue, designated as Fraction T-6, weighed 2783.1 gm.   It was reserved for the examination of other cell constituents.

Separation of a Highly Pigmented Fraction

It was mentioned earlier that emulsions formed during the extraction of the fat with ether.   When the emulsions were shaken with chloroform a dark red extract was obtained.   The chloroform extract was dried with sodium sulfate, filtered, and the solvent was distilled off in a current of carbon dioxide. A solid, dark red nearly black substance was obtained which weighed 1.7 gm.   It was easily soluble in chloroform, giving a red solution.   It dissolved in carbon disulfide with a dark red color.   In alcohol, ether, and acetic ester it was partly soluble giving a yellowish red color.   The solutions did not act as indicators.   The solid substance gave a green color with concentrated sulfuric acid.   This substance is designated as Fraction T-7.

Extraction of Bacterial Residue with Acid Alcohol

Bacteria which have been defatted by the mild methods used in this laboratory, *i.e.* extraction with a mixture of alcohol and ether followed with chloroform at room temperature, still contain a large amount of lipid.   The complete removal of all lipid requires drastic treatment of the bacteria with acids or with alkali but if the partly defatted bacteria are heated with alcohol containing a

small amount of hydrochloric acid a comparatively large proportion of the bound lipid is liberated and can be obtained by extraction with ether. This treatment also removes a large amount of water-soluble material from the bacterial cells.

In a preliminary experiment 10 gm. of the dried bacterial residue described above were refluxed for 4 hours with 200 cc. of a mixture of equal parts of alcohol and ether containing 1 per cent of hydrochloric acid. The cell residues were filtered off and the filtrate was concentrated *in vacuo* until the ether and most of the alcohol were removed. The residue which consisted of a fat-like emulsion was diluted with water and extracted twice with ether. The ethereal solution was dried with sodium sulfate and concentrated to dryness. A wax-like residue remained which weighed 0.893 gm. The aqueous solution was extracted with chloroform but on evaporation of the solvent only 0.033 gm. of material was obtained. The aqueous solution contained a slight amount of insoluble matter which after it had been filtered off, washed, and dried, weighed 0.114 gm. The aqueous solution was concentrated and the residue after drying *in vacuo* weighed 2.42 gm.

The operations described above yielded therefore about 34 per cent of soluble substances of which 9.2 per cent were lipids.

In order to secure some information regarding the nature of the ether-soluble material which was liberated on treatment with acid-alcohol, 170 gm. of the defatted bacteria were treated as described above, yielding about 8 per cent of an ether-soluble wax-like substance. This material was saponified and separated by means of the lead soap-ether treatment into solid and liquid fractions. The solid material obtained from the ether-insoluble lead soap was precipitated from hot alcohol and from acetone yielding a white amorphous powder. It melted at 60–61° and its neutralization value corresponded to a molecular weight of 607.

[The liquid portion obtained from the ether-soluble lead salts was a thick yellowish oil. In chloroform solution it showed no optical activity. It had an iodine number of 32.1 and its neutralization value corresponded to a molecular weight of 461.

It is evident from these data that the ether-soluble lipid contained but little if any ordinary fatty acids. The properties would indicate that it consisted largely of wax-like material.

Treatment of Cell Residue with Alkali

10 gm. of the cell residue, after the acid-alcohol treatment, were heated to boiling with 200 cc. of 5 per cent aqueous potassium hydroxide. A reddish brown solution resulted which contained

TABLE I

Substances Isolated from Timothy Bacillus

Fraction No.	Substance	Weight	Per cent
		gm.	
T-1	Phosphatide	18.7	0.59
T-2	Acetone-soluble fat	87.4	2.75
T-3	Polysaccharide	124.0	3.90
T-4	Chloroform-soluble wax	146.6	4.61
T-5	Yellow wax	11.8	0.37
T-6	Dried bacterial residue	2783.1	87.70
T-7	Pigment	1.7	0.05
Total weight from 1602 cultures..........		3173.3	100.00

TABLE II

Percentage of Lipoids from Acid-Fast Bacilli

	Type of organism			
	Human tubercle bacillus, Strain H-37	Avian	Bovine	Timothy
Approximate No. of cultures	2000	2000	1700	1600
Phosphatide.......................	6.54	2.26	1.53	0.59
Acetone-soluble fat...............	6.20	2.19	3.34	2.75
Chloroform-soluble wax...........	11.03	10.79	8.52	4.98
Total lipoids.....................	23.78	15.26	13.40	8.37
Polysaccharide...................	0.87	1.02	1.09	3.90
Dried bacterial residue............	75.01	83.71	85.50	87.70

only a small amount of a slimy insoluble material. The solution was cooled, filtered, acidified, and extracted with ether. The ether was evaporated leaving less than 1 per cent of a solid fat-like material.

A summary of the various fractions isolated from the timothy bacillus is given in Table I.

One of the objects of the present investigation was to compare a non-pathogenic acid-fast bacillus with pathogenic bacteria belonging to the same class.   The data presented in Table II show at a glance the variations in the lipoid content of the 4 acid-fast bacilli that have been extracted in this laboratory under similar conditions and with similar solvents.

The lipoid content of the avian and bovine strains is much less than that of the human, and the timothy bacillus has the least of all.   It will be noticed that as the lipoids decrease the amount of polysaccharide which passes into the alcohol-ether extract increases, the timothy bacillus yielding nearly 4 times as much as the other bacteria.

The various lipoid fractions herein described will be studied by chemical and biological methods as soon as possible in order to compare the chemical composition and the biological reactions with corresponding fractions isolated from tubercle bacilli.   Some preliminary experiments with the phosphatide have already been conducted in Dr. Sabin's laboratory at The Rockefeller Institute but the results have not yet been published.

In conclusion we desire to express our appreciation to Parke, Davis and Company who provided us with the bacteria.

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THE NUCLEIC ACID OF THE TIMOTHY BACILLUS

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INTRODUCTION

The timothy hay bacillus, *Mycobacterium phlei* (Moeller), is an organism which is classified by bacteriologists in the same general group with the tubercle bacillus, being an acid-fast, Gram-positive rod with the general appearance and growth characteristics of the latter organism. It differs markedly from the tubercle bacillus, however, in that it is entirely non-pathogenic. For that reason it was thought desirable, in connection with the research work sponsored by the National Tuberculosis Association (1), to determine in what respect it differs chemically from the tubercle bacillus. A proximate chemical analysis of the organism (2) and a study of its fat fractions (3) have already been reported upon in this *Journal.*

The present paper is concerned with the nucleic acid of the timothy bacillus and presents a striking difference between this organism and the tubercle bacillus, in that the latter posesses a nucleic acid of the animal type (4) whereas the timothy bacillus yields a nucleic acid ordinarily found associated with plants.

The bacteria used were grown on Long's synthetic medium (5), thus having only asparagine and ammonium citrate as sources of nitrogen, and the above two substances along with carbon dioxide and glycerol furnishing the carbon. The growth was carried on at a temperature of 37° in 1 liter Pyrex bottles placed on their sides, each containing a volume of 200 cc. of medium. At the end of approximately 4 weeks, the heavy pellicle was filtered off on large Buchner funnels, washed once with distilled water, and dried *in vacuo* at 40°.

EXPERIMENTAL

A. Preparation of Nucleic Acid—In order to procure the fat fraction for other work, the bacteria were successively extracted,

four times with a mixture of equal parts of 95 per cent alcohol and ether, and twice with chloroform. The final residue was then dried in the air and ground in a ball mill until it would pass a 50-mesh sieve. The material prepared in this manner lost 11.75 per cent of moisture and solvents when dried to constant weight in an Abderhalden drier at 100°.

The method used for the preparation of the nucleic acid represents a combination of conditions taken from several accepted methods for the preparation of yeast nucleic acid. One new feature, the use of colloidal ferric hydroxide, was introduced. This was borrowed, however, from Levene's method for preparing nucleic acid from animal organs (6). Three preparations were made, with 500 gm. of bacteria each time. The following procedure illustrates the method of preparation.

500 gm. of bacteria were ground into a paste with 2 liters of 2 per cent sodium hydroxide, this being done at room temperature. At the end of an hour, a liter of water was added and the resulting suspension of bacteria was neutralized to litmus with acetic acid and centrifuged. The resulting 1.75 liters of supernatant liquid were very cloudy. The residue was thoroughly suspended in 1.45 liters of water and again centrifuged, yielding 1.45 liters of extract. The combined solutions were treated with acetic acid as long as a precipitate formed. In order to obtain an effective filtration, a solution of colloidal ferric hydroxide[1] was now added. The precipitate was filtered off through fluted filter papers, the first liquid to run through having to be refiltered.

From this filtrate the nucleic acid was precipitated by the addition of hydrochloric acid to an acid reaction to Congo red, followed by 1 volume of 95 per cent ethyl alcohol. The flocculent precipitate was separated from its supernatant liquid as quickly as possible with a centrifuge and washed successively with 50 per cent, 95 per cent, and absolute alcohol, and twice with ether. The ether was then removed *in vacuo*.

The nucleic acid thus prepared (Sample C) was a pure white powder which dissolved in 0.1 N sodium hydroxide to form a perfectly clear but very slightly colored solution. It gave a strongly

[1] This was made from Merck's colloidal iron by diluting 30 cc. of a 5 per cent solution to 900 cc. It was found that a more flocculent precipitate was formed by the use of the very dilute iron solution.

positive test for pentose with orcinol. The biuret test as ordinarily performed was negative, but when done by the special technique of Osborne, a slightly positive reaction was obtained.[2]

In Table I are recorded the results of the three preparations. The moisture determination was made by drying a sample in an Abderhalden drier at 100° to a constant weight. The nitrogen, phosphorus, carbon, hydrogen, and guanine figures are calculated on a dry basis. The guanine determinations were made on 1 gm. samples according to Jones' directions for the isolation of guanine (7). The guanine obtained after hydrolysis of the nucleic acid was precipitated twice, including one treatment with norit, and

TABLE I

Analyses of Nucleic Acids

	Amount of bacteria	Yield of nucleic acid	Mois- ture	C	H	N	P	Gua- nine
	gm.	gm.	per cent	per cent	per cent	per cent	per cent	per cent
Sample A............	500	8.1	7.3	32.3	4.9	14.95	7.9	10.6
" B............	500	7.7	6.8	32.2	·5.0	14.95	7.95	10.65
" C............	500	8.1	5.2	32.9	5.2	14.7	7.35	11.25

finally weighed on a Gooch crucible after being dried in an oven at 120°.

B. Identification of Purines—The crude guanine obtained from the three guanine determinations was converted into guanine chloride and recrystallized from 5 per cent hydrochloric acid. For analysis the guanine chloride was dried in the air. The high nitrogen value found probably indicates a partial loss of water of crystallization.

$C_5H_5N_5O \cdot HCl \cdot 2H_2O$. Calculated, N 31.3; found, N 32.7

The filtrate from the guanine obtained in the quantitative determinations was worked up for adenine by precipitating the

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[2] Several years ago, the late Professor T. B. Osborne of the Connecticut Agricultural Experiment Station showed me an exceedingly delicate method of performing the biuret test. This is done by performing the test in the ordinary manner, then adding 10 or 20 drops of ethyl alcohol and a stick of solid potassium hydroxide. The alkali salts out the alcohol, which brings with it any of the biuret color present. By this modification distinctly positive biuret tests may be obtained on solutions which by the ordinary method appear to be negative.

purines as the cuprous salts and decomposing with hydrogen sulfide. The adenine was then precipitated as the picrate, 0.42 gm. being obtained. After recrystallization from 25 per cent acetic acid it melted at 290–294°.

$C_{11}H_8N_8O_7$. Calculated, N 30.65; found, N 30.45

C. Identification of Pyrimidines—4 gm. of nucleic acid were hydrolyzed in an autoclave at 150–160° for 5 hours with 20 cc. of 25 per cent sulfuric acid. After being cooled, the major portion of the sulfuric acid was removed with barium hydroxide and the purines precipitated with silver in dilute acid solution. The pyrimidines were then removed by the addition of more silver and barium hydroxide. The resulting precipitate was decomposed with hydrogen sulfide and the filtrate from the silver sulfide concentrated.

From this solution the cytosine was precipitated with picric acid. After twice recrystallizing the picrate, the cytosine was successively converted into the chloride, the silver salt, and finally into the free base. After two crystallizations it was dried in an oven at 120° for analysis.

$C_4H_5ON_3$. Calculated, N 37.8; found, N 37.3

After removal of picric acid from the cytosine picrate mother liquors, followed by concentration, uracil crystallized from the solution in its customary form. It was purified by several crystallizations from water and dried in an Abderhalden drier at 100°.

$C_4H_4O_2N_2$. Calculated, N 25.05; found, N 24.6

The color reaction for thymine, described by Harkins and Johnson (8), was performed on several occasions during the above described procedure, but in no case did it give a positive reaction. In order to make certain of the absence of this pyrimidine, the following experiment was performed.

The solution of partially hydrolyzed nucleic acid obtained from the pentose determination (see next paragraph), representing 1 gm. of nucleic acid, was evaporated to dryness *in vacuo* and taken up in 25 per cent sulfuric acid. It was subsequently hydrolyzed and the pyrimidine fraction isolated as previously described.

The solution was then concentrated and one-half of it tested for thymine by the method of Harkins and Johnson. Although this test is sensitive to 2 mg. of thymine in the presence of much larger quantities of cytosine, a completely negative test was obtained. This experiment also excludes the presence of 5-methylcytosine.

D. *Nature of the Sugar*—A determination of pentose was carried out on 1 gm. of nucleic acid Sample A by the method described by Browne (9). 0.177 gm. of furfural phloroglucide was obtained, representing, on the basis of moisture-free nucleic acid, 10.2 per cent of furfuraldehyde or 19.8 per cent of pentose, calculated from the average values given for xylose and arabinose.

DISCUSSION

The analytical results obtained upon the three samples of nucleic acid, although agreeing fairly well among themselves, indicate that the nucleic acid as used was not pure. This is to be regretted as it would have been desirable to have as pure a material as possible. However, owing to the expense involved in growing the bacteria, and due to previous experiences in trying to purify the nucleic acid wherein a great deal of material was lost with little improvement in purity, it was deemed advisable to proceed with the nucleic acid as originally prepared. It is thus fortunate that none of the main conclusions arrived at as a result of the research can reasonably be ascribed to the presence of an impurity.

Calculations, based on the volumes of liquid used and recovered in the preparation, show that the nucleic acid obtained represents a maximum of 80 per cent of what was actually present. Combining this figure with the actual yield, and correcting for the moisture contents of both the bacteria and the nucleic acid, we arrive at a figure of 2.15 per cent as the probable minimum amount of nucleic acid in the chloroform and alcohol-ether-extracted timothy bacillus.

Under the conditions used for the pentose determination, xylose and arabinose decompose into furfuraldehyde to the average extent of 80 per cent. Recently Hoffman (10) has reported conditions under which these two sugars decompose practically quantitatively. Increasing the furfuraldehyde value here reported by 25 per cent would raise it from 10.2 to 12.8 per cent, which compares favorably with the 14.0 per cent found by Hoffman for yeast nucleic acid. The percentages of guanine nitrogen, and

phosphorus are so high as to preclude the possibility that the furfuraldehyde, in the amount obtained, was derived from a carbohydrate impurity in the nucleic acid.

The absence of thymine and the presence of a pentose in the nucleic acid both lead to the conclusion that this substance is of the plant type, hitherto represented by yeast and triticonucleic acids.   This finding was contrary to expectations, as the tubercle bacillus, supposedly closely related to the timothy bacillus, has a nucleic acid of the animal type (4).   It is true that there is some question as to the advisability of referring to "plant" and "animal" nucleic acids, due to the fact that adenylic and inosinic acids are found in pancreatic and muscle tissues.   Thus Calvery (11) suggests that we refer to "pentose" and "hexose" nucleic acids. Very recently, however, Levene and his coworkers (12) have shown that the sugar in thymus nucleic acid is d-2-ribodesose rather than a hexose.   Furthermore, Embden and Schmidt (13), having noticed that an enzyme preparation which deaminized adenylic acid from muscle failed to act upon adenylic acid from yeast, found that the two varieties, previously thought to be identical, are in reality two different substances.   Although their melting points when taken individually are the same, when mixed there is a marked depression.   Embden and Schmidt also noted other physical and chemical differences.   If adenylic acid from muscle is not identical with that of yeast, it removes the main objection to the terms animal and plant nucleic acids.

The finding of a difference between the nucleic acids of the timothy and tubercle bacilli leads one to wonder if it is in any way connected with the difference in their pathogenicity.   That, however, would be a very difficult question to decide.   One that would be more definitely within the grasp of the chemist has to do with the classification of bacteria.   As the matter now stands, bacteria of all kinds are allocated to various groups, the almost sole basis of classification being their external appearances and staining reactions.   The timothy and tubercle bacilli have been thus grouped together.   However, it would seem that a difference in chemical make-up as wide as that described in this paper would furnish a much more fundamental basis for classification than the older method.   At the present time work is progressing in this laboratory to determine the types of nucleic acids in a series of

widely differing bacteria in order to determine whether it is possible to separate them into at least two groups, and thus furnish a step in the chemical classification of bacteria.

SUMMARY

The nucleic acid has been extracted from the timothy hay bacillus, *Mycobacterium phlei* (Moeller), to the extent of a little over 2 per cent. The elementary analysis indicates that it has the approximate composition of other nucleic acids.

Upon hydrolysis the nucleic acid of the timothy bacillus yields guanine, adenine, uracil, and cytosine, but no thymine. It contains at least 20 per cent of pentose.

The presence of pentose and the absence of thymine lead to the conclusion that we are here dealing with a plant nucleic acid, in sharp distinction to the supposedly closely related tubercle bacillus which contains a nucleic acid of the animal type.

The possibility of a chemical classification of bacteria has been suggested.

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THE MANGANESE METABOLISM OF THE RAT*

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Since the recognition of the general occurrence of small amounts of certain mineral elements in plant and animal materials and the discovery of particular rôles in which some of these elements function, a new interest in them has arisen. Of the elements whose functions have not as yet been ascertained, manganese has received considerable attention and numerous hypotheses concerning its value to plants and animals have been offered.

In 1924, Levine and Sohm (1) reported that manganese in amounts equivalent to 1 part in from 2,000 to 10,000 parts of ration improved the appearance of rats, increased their activity, and made the offspring grow faster than the controls. Richet, Gardner, and Goodbody (2) demonstrated that small amounts of manganese produced a favorable effect upon the growth of dogs, if administered every 3 days, but unfavorable, if given more often. McHargue (3), working with rats, and Bertrand and Nakamura (4), working with mice, noted a favorable effect of manganese upon the growth of these animals. McCarrison (5) found that doses of 0.009 mg. per rat daily accelerated growth whereas 0.56 mg. tended to retard it. The discovery by Hart, Steenbock, and associates (6) that copper is an active agent in hemoglobin formation has directed attention to other mineral elements and suggested the possibility that some of these elements may share with copper in the regeneration of hemoglobin. These investigators (7, 8) submit evidence that copper alone functions in this capacity. On the other hand, Titus and associates (9, 10) report that manganese gives almost as good results as copper. Likewise,

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Beard and Myers (11, 12) believe that a number of elements, manganese included, may take part in the performance of this important function. Faludi (13) reports that manganese administered in the form of an albuminate exerts a marked regenerative action on the blood. Alone and in combination with arsenic, manganese has been used by him in the clinical treatment of both secondary and pernicious anemia. Although there is not unanimity among investigators in assigning specific functions to manganese, there is a general belief that this element plays an important rôle in the animal organism.

The primary object of this investigation was to make a more detailed and coordinated study of the metabolism of this element than has heretofore been reported.

<center>EXPERIMENTAL</center>

The experiments consisted of: (1) determination of the manganese content of the whole animal at different ages when given a complete ration and when this was supplemented with manganese, (2) analysis of the various tissues of rats receiving a complete ration with and without manganese additions, (3) a study of the metabolism of manganese by animals on a high and low intake of the element, and (4) determination of the manganese content of the whole animal on an incomplete ration and on the same ration when supplemented with manganese or combinations of this element with copper and iron.

Preparation of Samples—All animals taken for analysis were carefully washed with distilled water at least three times to remove any traces of adhering manganese. The intestines were removed from all animals 21 days old or over, and the bodies were dried in a steam oven at 100°. The dried samples were then stored in glass jars until the analyses could be made.

Methods of Analysis—The manganese determinations, with the exception of a few in which the official method (14) was used, were run according to the procedure for manganese in animal materials as given in a previous publication (15). The number of animals required for a determination necessarily varied with the size. A single analysis required, as a rule, two litters at birth, one litter at 12 days, two animals at 21 days, and one animal at 70 and 180 days. In the case of the larger animals, aliquots of the acid solution afforded sufficient manganese for the determinations.

Blood samples were taken according to the method described by Mitchell and Schmidt (16), and the hemoglobin determinations were made with the Newcomer hemoglobinometer.

Manganese Content of Animals Receiving a Stock Ration as Related to Age and Manganese Intake—In this series of experiments determinations were made of the manganese content at five different ages of rats which had received the stock ration with and without manganese additions. The analyses were made (1) at birth in order to reveal the extent to which manganese is transmitted through the placenta, (2) at 12 days for an indication of the effect, if any, of the manganese content of the mother's milk, because at that age the animal has not opened its eyes nor had access to solid food, (3) at 21 days, which represents the approximate weaning age, (4) at 70 days, the breeding age, and (5) at 180 days, full maturity.

The control animals received no manganese other than that contained in the stock ration described by Waddell and Steenbock (17). The manganese-fed animals received a stock ration, essentially the same as the first, plus manganese at levels which varied with the age of the animal. In this ration dried milk comprised one-third of the food consumed. Previous records in this laboratory have shown that on the Steenbock stock ration (18) about one-third of the solids consumed by the animals comes from whole milk, when this is fed *ad libitum*. The substitution of dried milk for the whole milk in the Steenbock ration was made because it was believed that the manganese intake per animal could be more evenly regulated by employing a dry ration. As thus compounded it contained 10.47 mg. of manganese per kilo and consisted of the following.

	per cent
Yellow corn	50.0
Klim milk powder	33.4
Linseed oil meal	10.5
Crude casein	3.5
Alfalfa	1.4
NaCl	0.4
Bone ash	0.7

When additional manganese was incorporated into the ration, this was done by evaporating a solution of manganese sulfate on a

portion of the feed. After the material had dried thoroughly, it
was pulverized with a mortar and pestle and incorporated into
the remainder of the ration at the time of feeding. Animals
between weaning age and 70 days were given only 1 mg. of added
manganese daily, but thereafter the amount was increased to 5 mg.

The control animals were obtained from the stock colony at the
ages specified. The animals receiving additional manganese were
the offspring of twelve females and two males which, prior to being
mated, were placed on the milk powder ration plus 5 mg. of man-

TABLE I

Manganese Content of Rats at Different Ages on a Low Intake of Manganese
The stock ration contained liquid milk.

Age of animals	No. of animals	Moisture		Dry matter per animal		Mn per animal		Mn in dry matter, average	Hemoglobin	
		Limits	Average	Limits	Average	Limits	Average		Limits	Average
days		*per cent*	*per cent*	*gm.*	*gm.*	*mg.*	*mg.*	*mg. per kg.*	*gm. per 100 cc.*	*gm. per 100 cc.*
0	103	85.2–86.6	85.9	0.64–1.04	0.84	0.0013–0.0019	0.0015	1.76		
12	25	71.6–75.1	73.1	4.96–9.58	7.19	0.0053–0.0093	0.0065	0.90		
21	18	68.6–71.3	70.1	10.3–13.6	12.1	0.0114–0.0174	0.0147	1.22	7.3–11.6	9.1
70	11	57.6–67.4	63.2	55–106	72.2	0.0444–0.0924	0.0595	0.82	14.7–17.6	16.0
180	9	61.8–64.9	63.9	73–142	91.3	0.0505–0.0850	0.0676	0.73	12.1–15.3	14.3

ganese per animal daily. All animals, except when it was desired
to obtain young for analysis at birth, were kept in cages provided
with shavings. To prevent the young analyzed at birth from
nursing, the mothers were placed on ½ inch mesh screens 2 or 3
days before parturition. In order that the animals might receive
proper nourishment the number of young per litter was reduced to
six about 1 week after birth.

In Table I are shown the data obtained from the analyses of
rats grown on the stock ration. These figures show that most of
the manganese was stored before the animals reached sexual

maturity; the amount at birth was only 0.0015 mg. per animal, but this reached an average of 0.0595 mg. by the time the animals were 70 days of age. The maximum for any group was attained by the 180 day animals—0.0676 mg. each—although the upper limit for this group was somewhat lower than that for the 70 day animals. The storage of the element during the first 12 days was at the rate of 0.0004 mg. per day whereas, during the two following periods, the average daily gain in each instance was 0.0009 mg.

TABLE II

Manganese Content of Rats at Different Ages on a High Intake of Manganese

The stock ration contained milk powder plus manganese.

Age of animals	No. of animals	Moisture		Dry matter per animal		Mn per animal		Mn in dry matter, average	Hemoglobin	
		Limits	Average	Limits	Average	Limits	Average		Limits	Average
days		*per cent*	*per cent*	*gm.*	*gm.*	*mg.*	*mg.*	*mg. per kg.*	*gm. per 100 cc.*	*gm. per 100 cc.*
0*	100	85.9–86.7	86.2	0.68–0.88	0.79	0.0018–0.0023	0.0021	2.61		
12*	29	73.2–75.4	74.2	5.5–7.8	6.2	0.0044–0.0086	0.0064	1.03		
21*	19	70.3–75.3	73.5	5.2–9.1	7.4	0.012–0.0183	0.0156	2.12	4.6–10.9	7.8
70*†	11	66.8–73.0	69.0	29–61	43.3	0.0604–0.0952	0.0754	1.74	11.1–14.3	12.7
180*†‡	11	62.6–67.7	66.3	58–104	84.2	0.088–0.149	0.1130	1.34	11.6–15.3	12.9

* Mothers received 5 mg. of added manganese daily.

† Animals received 1 mg. of added manganese daily from 21 to 70 days.

‡ Animals received 5 mg. of added manganese daily from 70 to 180 days.

A daily storage of only 0.00007 mg. per animal took place between 70 and 180 days.

If the concentration of manganese in rat milk is as low as in cow's milk, the slow rate of storage during the first 12 days is easily explained. Inasmuch then as the young animals later consumed some of the mother's ration, a marked rise in manganese content was to be expected between the 12th and the 21st days. On the dry basis, the highest concentration, 1.76 mg. per kilo, occurred at birth. During the period when milk formed the sole

food a decrease ensued, but a second high peak, 1.22 mg. per kilo, was reached at 21 days. A second decline then began and continued through the two remaining periods.

In Table II are presented the data for the young produced from mothers receiving a high level of manganese and for animals that received added manganese during their whole span of life. These animals showed a marked storage of the element before attaining sexual maturity as did the animals receiving only the stock ration. However, they differed from the latter in that they continued to store the element at an appreciable rate between 70 and 180 days. The average daily gain for each period was: (1) 0.0004 mg., (2) 0.0010 mg., (3) 0.0012 mg., and (4) 0.0003 mg.; the storage is seen to be especially pronounced during the second and third periods (12 to 70 days).

Upon comparing Tables I and II, it will be noted that the manganese content at birth was 40 per cent higher when the mothers received 5 mg. of manganese daily. This indicates increased transmission of the element through the placenta,—a process, which apparently does not take place in the case of added copper (19). That the manganese content of milk was affected very slightly, if at all, by feeding additional amounts of the element is indicated when comparison is made of the storage by the two groups during the first 12 days of life. The controls contained 0.0065 mg. as compared with 0.0064 mg. in the manganese-fed animals. However, it will be noted that the animals in the second group were somewhat smaller than those in the first. Growth of the animals in the second group was also distinctly retarded at 21 and 70 days, but at 180 days they had very nearly reached the size of the stock animals. An attempt was made to learn whether the added manganese or the absence of liquid milk was responsible for this poorer growth. For this study some of the breeding stock were placed on the ration containing liquid milk and were given manganese in the same concentrations as had previously been added. Prior to the shift from the dry ration the females had begun to show an inability to nourish the young properly, and high fatalities often resulted. This difficulty continued on the ration supplemented with liquid milk, and, consequently, the data are not so conclusive as would be desired. Three litters of three, three, and five young, respectively, when 21 to 23 days of age weighed an average of 45

gm. per animal. Eight of these animals, which after weaning were placed on this ration plus 1 mg. of manganese per animal daily, attained an average weight of 179 gm. at 55 days of age, a weight essentially the same as that of the controls at the same age. These results suggest that the absence of liquid milk rather than the presence of the added manganese was the cause of the retarded growth on the manganese-dry ration. No final conclusion can be drawn on this point, however, until more data are available.

Hemoglobin values for the control rats were slightly higher at all ages than for the animals receiving the milk powder ration. Whether this was due to the presence of manganese in the milk powder ration or to the ration itself has not been determined. A similar depression of the hemoglobin values has been observed when this ration with copper additions was given to rats (19). No other favorable or unfavorable effects of manganese upon the young were noted. However, as has been mentioned previously, the females receiving additional manganese failed to suckle their young well after the production of several litters. They continued to produce young normally throughout the 8 months during which they were bred, but toward the end of this period in some instances the entire litter would be starved to death before the end of 12 days. In other instances varying numbers of the six would survive, but these were seldom uniform in weight, the largest sometimes weighing 2 or 3 times as much as the smallest. Some of the offspring were bred and were found to produce normal young, which they suckled very well for a time. After having reared only one or two litters, they too ceased to nourish the young normally. Their failure to rear successfully as many litters as their mothers is possibly to be attributed to their having been subjected to a high intake of manganese throughout life. Further data need to be obtained.

Distribution of Manganese in Tissues—Since the manganese content of the body as a whole had been shown to vary markedly when manganese was added to the ration, a study was made of its storage in various tissues. For this study ten rats, approximately 3 months of age, were fed the modified stock ration plus 5 mg. of manganese per animal daily for 91 days while another group of eight animals received only the stock ration. At the conclusion of the feeding experiment the animals were thoroughly washed

with distilled water and, after removal of the intestinal tract, the bodies were dissected. Since it was impossible to free the tissues completely from blood, no attempt was made to remove any of it. As a matter of fact, contamination of tissues with blood could not have led to a great error because analysis of blood showed that the concentration on a normal intake of the element was only 0.17 mg. per liter. After dissection the tissues were dried in a steam oven at 100° and analyzed. With the exception of bone, hide, and muscle, single analyses were made on composite samples. Since

TABLE III

Distribution of Manganese in Tissues of Adult Rats on a Low Intake of Manganese

The stock ration contained liquid milk.   Age of animals, about 6 months.

Tissue	Mois- ture	Sample (8 animals)		Mn per animal	Mn in dry matter
		Weight, dry	Mn		
	per cent	gm.	mg.	mg.	mg. per kg.
Bone............................	51.6	205.0	0.1613	0.0202	0.79
Brain...........................	77.0	3.3	0.0067	0.0008	2.03
Heart...........................	76.5	2.3	0.0056	0.0007	2.43
Hide............................	57.7	332.0	0.1544	0.0193	0.47
Kidney..........................	73.3	6.0	0.0164	0.0021	2.73
Liver...........................	73.2	39.4	0.2424	0.0303	6.15
Lung............................	79.3	4.5	0.0059	0.0007	1.31
Muscle..........................	69.6	357.0	0.1985	0.0248	0.56
Spleen..........................	76.1	2.2	0.0053	0.0007	2.41
Totals.........................		951.7	0.7965	0.0996	0.84

large amounts of the three tissues mentioned were available, triplicate determinations, one of which involved the recovery of a known amount of added manganese, were made on each of them. This procedure was essential in the analysis of bone because, when the solution was boiled for oxidation, a precipitate formed. Even with repeated extractions of such precipitates one cannot be assured of the reliability of the determination unless a good recovery of added manganese is obtained. Recoveries averaging 93.4 per cent were obtained in these analyses.

Table III, which presents the data for rats receiving no man-

ganese other than that in the stock ration, shows that the liver contained the largest amount and was followed in descending order by the muscles, the bones, the hide, and the kidneys. The amounts in the remaining tissues were very small and approximately equal.

When manganese was added to the ration, the quantity in the bones was raised from third to first place, and this was followed by the liver, the hide, the muscles, and the kidneys, in the order named (see Table IV). The bones of the manganese-fed animals contained 191 per cent more manganese than those of the stock

TABLE IV

Distribution of Manganese in Tissues of Adult Rats on a High Intake of Manganese

The stock ration contained milk powder plus 5 mg. of manganese per animal daily. Age of animals, about 6 months.

Tissue	Moisture	Sample (10 animals)		Mn per animal	Mn in dry matter
		Weight, dry	Mn		
	per cent	*gm.*	*mg.*	*mg.*	*mg. per kg.*
Bone...........................	53.3	265.0	0.5865	0.0587	2.21
Brain..........................	76.6	4.0	0.0131	0.0013	3.28
Heart..........................	77.4	2.9	0.0069	0.0007	2.38
Hide...........................	56.6	412.0	0.3461	0.0346	0.84
Kidney.........................	72.7	8.0	0.0265	0.0027	3.31
Liver..........................	73.4	49.6	0.3922	0.0392	7.91
Lung...........................	78.1	6.2	0.0072	0.0007	1.16
Muscle.........................	71.6	392.0	0.23	0.023	0.59
Spleen.........................	73.7	3.0	0.0059	0.0006	1.97
Totals.........................		1142.7	1.6144	0.1614	1.41

animals. The amounts in the hide, liver, and kidneys likewise showed increases of 79, 29, and 29 per cent, respectively. The concentration in muscle of the manganese-fed animals was about 5 per cent higher although there was a decrease in the total amount due to a slight difference in the size of the rats in the two groups. The data indicate no change in the heart, lung, and spleen but an appreciable increase for the brain. The figures for the brain are subject to question since the amount of manganese present in the

sample was somewhat less than that needed for an accurate analysis.

The chief centers of storage are therefore bone, hide, muscle, and liver. That the first three named should contain much of the manganese of the body is to be expected for they constitute a

TABLE V

Balance Experiment on Low and High Intake of Manganese (Rat 1)

Date	Dried feces	Feces Mn	Urine Mn	Part of total Mn in feces	Intake Mn	Output Mn	Balance
Pre-manganese period							
1930	*gm.*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*
Mar. 19–24...............	11.8	0.70	0.16	81.4	0.75	0.86	−0.11
" 25–30...............	11.2	0.60	0.15	80.0	0.75	0.75	0
Transition period							
Mar. 31–Apr. 5...........	10.7	20.9	0.25	98.6	30.8	21.2	+9.6
Manganese period							
Apr. 6–11...............	10.9	24.1	0.32	98.8	30.8	24.4	+6.4
" 12–17...............	11.3	26.1	0.31	98.9	30.8	26.4	+4.4
Transition period							
Apr. 18–23...............	11.1	2.8	0.18	93.3	0.75	3.0	−2.3
Post-manganese period							
Apr. 24–29...............	10.7	0.65	0.16	80.2	0.75	0.81	−0.06
" 30–May 5...........	10.1	0.68	0.16	81.0	0.75	0.84	−0.09
May 6–11...............	11.5	0.63	0.18	77.7	0.75	0.81	−0.06
" 12–17...............	10.4	0.60	0.19	76.0	0.75	0.79	−0.04

large proportion of the body weight. Muscle, for example, which makes up about 35 per cent of the body weight, contained 25 per cent of the manganese. The liver, on the other hand, represents only about 4 per cent of the body weight but contained 30 per cent of the element.

Since bone had been shown to be an important tissue for the storage of manganese, an attempt was made to obtain some sug-

gestion as to where the element is stored.   Analyses were made on the marrow, shaft, and head of a soup bone obtained at a local market.   Only a trace of the element was found in a 9 gm. sample of marrow but concentrations of 0.38 and 0.34 mg. per kilo on the fresh basis were obtained for the shaft and head, respectively. Further work should be done to determine in what portion of the bone the manganese is stored by an animal on a high intake of the element.

Excretion of Manganese—In this experiment an attempt was made to follow the excretion of manganese by three adult male rats with and without the addition of manganese to the milk powder ration.   The same technique was employed in the care of the

TABLE VI

Summary of Data on Excretion of Manganese

Excretory product and period (6 days)	Rat 1	Rat 2	Rat 3
	mg. of Mn	*mg. of Mn*	*mg. of Mn*
Urine			
Pre-manganese periods, average.	0.16	0.15	0.15
Manganese " " .	0.32	0.34	0.19
Post-manganese " " .	0.17	0.18	0.19
Feces			
Pre-manganese periods, average.	0.65	0.62	0.68
Manganese " " .	25.1	23.3	25.9
Post-manganese " " .	0.64	0.68	0.67

rats, and the same periods were observed as in the study of the excretion of copper (19).

The data for the three animals were essentially the same in character and only those for one will be given in detail (see Table V). The most significant of the data in Table V together with those obtained on Rats 2 and 3 are summarized in Table VI and form the basis for the following conclusions.   By the addition of manganese to the milk powder ration the quantity of the element excreted in the urine by Rats 1 and 2 was doubled, but the urine value for Rat 3 was increased only about 25 per cent.   The major portion of the added manganese was excreted in the feces as is indicated by an average manganese content of 25 mg. during 6 days of the manganese period as contrasted with an average of

about 0.65 mg. during the pre- and post-manganese periods. Approximately 80 per cent of the manganese appeared in the feces during a normal intake of the element, but this was increased to 99 per cent when additional manganese was included in the ration.

Unfortunately, the data given in Tables V and VI do not represent the absolute values because only about 82 per cent of the manganese intake was accounted for in the excreta. An investigation into the cause of this discrepancy revealed that in such high concentrations manganese is not entirely recovered by the phosphoric acid extraction. This was not discovered until all of the material had been used for analysis and, consequently, it was impossible to correct the results by making a second series of determinations using hydrochloric acid for extraction according to the official method (14). However, the results as given in these tables indicate in a general way, at least, how manganese is excreted by the animal organism.

Manganese Content of Animals Receiving a Milk Ration as Related to Intake of Manganese, Copper, and Iron—Since the ration used in the preceding experiments afforded sufficient quantities of all mineral elements for proper nutrition, it seemed desirable to study the storage of manganese on a ration known to be deficient in some of these elements. Consequently, whole milk *ad libitum* was chosen as the basal diet for such studies. Five groups of young rats from the stock colony, having an average weight, when 21 days of age, of 42, 43, 45, 43, and 38 gm. respectively, were placed in cages provided with shavings and were supplied with whole milk *ad libitum* over a period of 7 weeks. The milk was produced by the university herd on a winter ration and was found to have an average manganese content of about 0.02 mg. per liter. The first group received no additions, the second received manganese, the third manganese and copper, the fourth manganese and iron, and the last all three of these elements. The manganese, copper, and iron, when added to the ration, were fed at the rate of 1.0, 0.05, and 0.5 mg., respectively, per animal daily. Solutions of the first two were prepared by dissolving the c.p. grade of the corresponding sulfates in water. The iron solution was made according to the method used in its preparation for work on anemia (20). Consumption of all the minerals given was effected by adding them to only a small quantity of milk in the morning, and later

in the day, when this had been consumed, an excess of milk was placed in the feed receptacles.

Upon comparison of the weights of the animals in Table VII it will be noted that the average weight, dry basis, for the group receiving only whole milk was 23.9 gm., whereas, it was much greater for all of the other groups, a maximum of 52.7 gm. being reached

TABLE VII

Manganese Content of Rats on Milk with and without a Manganese Supplement

No. of animals	Moisture		Dry matter per animal		Mn		Mn in dry matter (average)	Hemoglobin	
	Limits	Average	Limits	Average	Limits	Average		Limits	Average
Group I. Whole milk									
	per cent	per cent	gm.	gm.	mg.	mg.	mg. per kg.	gm. per 100 cc.	gm. per 100 cc.
12	64.7–69.6	68.2	17.5–28.4	23.9	0.024–0.043	0.032	1.33	2.8– 7.0	4.6
Group II. Whole milk + 1 mg. Mn per animal daily									
12	67.1–70.5	69.1	22.9–37.4	31.0	0.075–0.124	0.101	3.28	4.0–11.0	7.9
Group III. Whole milk + 1 mg. Mn + 0.05 mg. Cu per animal daily									
7	66.0–70.8	68.1	46.0–62.0	52.7	0.059–0.089	0.074	1.40	12.1–14.9	13.1
Group IV. Whole milk + 1 mg. Mn + 0.5 mg. Fe per animal daily									
7	66.7–69.3	68.1	39.0–51.0	46.0	0.067–0.090	0.081	1.77	6.0–11.6	9.8
Group V. Whole milk + 1 mg. Mn + 0.05 mg. Cu + 0.5 mg. Fe per animal daily									
12	65.6–69.1	67.5	34.3–53.0	43.5	0.059–0.089	0.076	1.73	11.4–16.1	13.1

by the animals receiving manganese and copper. Milk consumption was greater when manganese alone or in combination with the other elements was added and better growth was obtained. The animals in Group III, which received added manganese and copper, were larger than those in Group V which received iron also. This unexpected result may be due to the larger initial weight

(7 gm.) of the animals in Group III, and to the fact that the experiments were not run simultaneously.

Table VII shows that the animals in Group I contained 0.032 mg. of manganese at 70 days of age as compared with 0.0147 mg. at 21 days, the age at which they were placed on the whole milk diet (Table I). Consumption records indicated that the total amount of milk ingested was close to 1.5 liters per animal. Since this quantity of milk contained about 0.03 mg. of manganese, approximately 60 per cent of the element in the ration was stored. The animals receiving manganese as the only addition stored the element to a striking degree. At 70 days they contained 0.101 mg., the highest value obtained for any animals of this age although they were not so large as many of the animals in Groups III to V. When copper and iron, either singly or together, were added to the manganese-milk ration, the accumulation of manganese was not so marked. Decreases in storage of the element resulting from the addition (1) of copper, (2) of iron, and (3) of a combination of the two to the manganese-milk ration were 27, 20, and 26 per cent, respectively.

Although these experiments were not intended to deal primarily with the subject of anemia, the data obtained in this connection should be noted. The final concentration of hemoglobin in the blood of animals receiving manganese as the only addition was somewhat higher than when the animals received only whole milk. The addition of iron to the manganese-milk ration had little effect, but when copper was added to this ration, the hemoglobin values were as high as when a combination of the three elements was added to the milk ration. In this connection it should be remembered that the animals had access to their feces and some of the effects may have been due to utilization of the minerals excreted therein. Moreover, since the manganese and copper salts were not purified, they may have contained traces of iron sufficient to effect the elevation.

<center>SUMMARY</center>

1. On a stock ration the total amount of manganese in the rat increased from 0.0015 mg. at birth to 0.0676 mg. at 180 days of age. The concentration was highest at birth, decreased during the first 12 days, rose to the second highest peak at 21 days, and decreased

thereafter.   Most of the manganese stored by the adult animal was found in the body at 70 days; *i.e.*, at the time of sexual maturity.

2. The manganese content of the rat at birth was raised 40 per cent by subjecting the mother to a high intake of the element during the gestation period.

3. When the young received only the mother's milk (0 to 12 days), the storage of manganese was slow.   Additions of manganese to the mother's ration did not increase the storage during this period.

4. The addition of the element to the ration effected a marked increase in the manganese content of the body at 21, 70, and 180 days.

5. Mothers on a high intake of the element were unable, after rearing several litters, to properly nourish their young.

6. The chief storage tissues for the element are liver, muscle, bone, and hide.   By the addition of manganese to the ration the amounts of the element in bone, hide, liver, and kidney were raised 191, 79, 29, and 29 per cent, respectively.   The concentration, however, was highest in the liver.

7. Adult rats on the stock ration excreted 80 per cent of the manganese in the feces.   When the ration was supplemented with 5 mg. of manganese daily, this proportion was raised to 99 per cent.

8. Young rats on a whole milk diet retained approximately 60 per cent of the manganese ingested over a period of 7 weeks.

9. Rats receiving milk plus manganese showed a marked retention of the element.   Although they weighed only one-third more than those on milk alone, they contained three times as much manganese.

10. Additions of copper and iron, either singly or together, to a manganese-milk ration decreased the retention of manganese.

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CONFIGURATIONAL RELATIONSHIPS OF PHENYLATED CARBINOLS. III*

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In a recent publication, Levene and Stevens[1] correlated by direct chemical methods the configuration of ethyl-β-phenethyl carbinol with that of the aliphatic carbinols and with ethyl-β-cyclohexethyl carbinol. The relationship of their rotations was then made the basis of correlating ethylbenzyl carbinol with the aliphatic carbinols. The conclusions regarding the latter relationship were given in a provisional way until such time as it could be established by direct chemical methods. A direct chemical way has now been found for the correlation of the configuration of methylbenzyl carbinol with aliphatic secondary alcohols and the method will be applied to other phenylated and to branched chain carbinols. The method is based on the condensation of optically active ethylenic oxides with aliphatic or aromatic radicals by the Grignard reaction, a method employed by Henry[2] and by Fourneau and Tiffeneau,[3] for similar purposes.

Theoretically, two reactions are possible, one leading to the secondary and the other to the primary alcohol.

$$R_1CH_2\text{—}CHOH\text{—}R \leftarrow R_1MgBr + H_2C\underset{\diagdown\diagup O}{\text{———}}CH \cdot R \rightarrow HO \cdot CH_2\text{—}\underset{\underset{R_1}{|}}{CH}\text{—}R$$

$$\text{I} \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \text{II}$$

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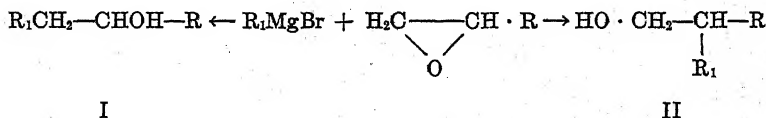
* Papers I and II of this series are those by Levene, P. A., and Stevens, P. G., *J. Biol. Chem.*, **87**, 375 (1930); **89**, 471 (1930) respectively.

[1] Levene, P. A., and Stevens, P. G., *J. Biol. Chem.*, **87**, 375 (1930).

[2] Henry, L., *Compt. rend. Acad.*, **145**, 453 (1907).

[3] Fourneau and Tiffeneau, *Compt. rend. Acad.*, **145**, 437 (1907).

81

In the case of condensation with propylene oxide, the reaction leads to the secondary carbinols (I), according to the experience of Henry and of Fourneau and Tiffeneau.   Nevertheless, it seemed desirable to verify this conclusion.

Besides, it was shown by Levene and Walti[4] that optically active propylene oxide hydrolyzes to the corresponding glycol with or without a Walden inversion depending upon the conditions of the reaction.   A priori, it is possible that a Walden inversion may accompany also the reaction of condensation.   Hence, the correlation of the configurations of the parent oxide and of the resulting carbinol depends upon the occurrence or non-occurrence of the Walden inversion.

Thus, in order to apply the above reaction for the correlation of secondary alcohols to that of propylene oxide, it was necessary to demonstrate first that the above reaction proceeded in a way leading to secondary carbinols and second, that the reaction took place without Walden inversion.   Both questions could be answered by condensing propylene oxide of a known configuration with methyl-, ethyl-, or propylmagnesium bromide.   The carbinol thus obtained could be easily identified and if it happened to be a secondary carbinol, its configuration could be determined by the direction of its rotation.   With this aim in view, dextro-propylene oxide was condensed with propylmagnesium bromide.   The carbinol obtained boiled at 136–138°, and hence, was the secondary carbinol inasmuch as the boiling point of the primary carbinol given by different authors differs from 146.5° to 148° and the boiling point of the secondary carbinol is given at 136° by Sabatier and Senderens,[5] at 138–139° by Zelinsky and Przewalski,[6] and at 139.5° by Ponzio.[7]   The carbinol obtained by us rotated to the left and this fact indicated that the reaction proceeded without Walden inversion, as may be seen from the following set of figures.

[4] Levene, P. A., and Walti, A., J. Biol. Chem., 73, 263 (1927).

[5] Sabatier, P., and Senderens, J. B., Compt. rend. Acad., 137, 302 (1903).

[6] Zelinsky, N., and Przewalski, E., Chem. Zentr., 2, 1855 (1908).

[7] Ponzio, G., Gazz. chim. ital., 31, 1, 404 (1901).

$$\begin{array}{cccc}
\text{COOH} & \text{CH}_2\text{OH} & \text{CH}_2 & \text{CH}_2-\text{C}_3\text{H}_7 \\
| & | & |\diagdown \\
| & | & |\text{O} \\
| & | & |\diagup & | \\
\text{HCOH} \rightarrow & \text{HCOH} \rightarrow & \text{HC} \xrightarrow{+\ \text{C}_3\text{H}_7\text{MgBr}} & \text{HCOH} \\
| & | & | & | \\
\text{CH}_3 & \text{CH}_3 & \text{CH}_3 & \text{CH}_3 \\
\text{Levo} & \text{Levo} & \text{Dextro} & \text{Levo}
\end{array}$$
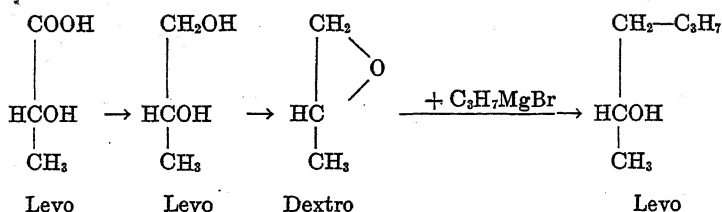
III

In this set of substances the configurations had been established by previous work of Levene and Haller[3] and Levene and Walti.[4] The methylbutyl carbinol, the propylene glycol, and the propylene oxide are each correlated to lactic acid in an identical manner.

With this information then, it was possible to apply the above method of synthesis for the elucidation of the configuration of methylbenzyl carbinol. In this case again the reaction, *a priori*, may lead to a primary and to a secondary alcohol. However, on oxidation the carbinol obtained by us was converted into methylbenzyl ketone. From the figures below it can be seen that the secondary carbinol leads to methylbenzyl ketone, whereas the primary carbinol on oxidation forms methylphenyl acetic acid.

$$\text{CH}_3 \cdot \text{CHOH} \cdot \text{CH}_2\ \text{C}_6\text{H}_5 \rightarrow \text{CH}_3\ \text{COCH}_2\ \text{C}_6\text{H}_5$$

IV

$$\begin{array}{c}
\text{CH}_3 \cdot \text{CH} \cdot \text{CH}_2\text{OH} \rightarrow \text{CH}_3 \cdot \text{CH} \cdot \text{COOH} \\
|| \\
\text{C}_6\text{H}_5\text{C}_6\text{H}_5
\end{array}$$
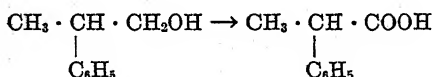
V

Again, the condensation of dextro-propylene oxide led to a levo-methybenzyl carbinol and hence levo-methylbenzyl carbinol should be correlated to levo-lactic acid and to dextro-propylene oxide in the following way.

[3] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **67**, 329 (1926); **79**, 475 (1928).

$$
\begin{array}{cccccc}
\text{COOH} & & \text{CH}_2\text{OH} & & \text{CH}_2 & & & \text{CH}_2-\text{C}_6\text{H}_5 \\
| & & | & & | \diagdown\text{O} & & & | \\
\text{HCOH} & \rightarrow & \text{HCOH} & \rightarrow & \text{HC} \diagup & \xrightarrow{\text{C}_6\text{H}_5\text{MgBr}} & & \text{HCOH} \\
| & & | & & | & & & | \\
\text{CH}_3 & & \text{CH}_3 & & \text{CH}_3 & & & \text{CH}_3 \\
\text{Levo} & & \text{Levo} & & \text{Dextro} & & & \text{Levo}
\end{array}
$$
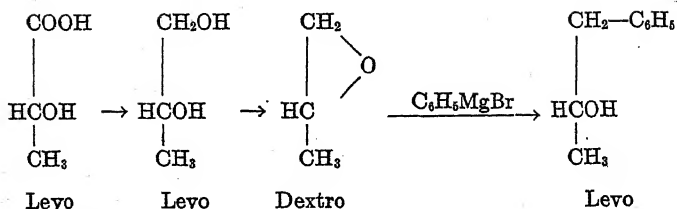
<div align="center">VI</div>

Thus, the conclusion regarding the configuration of levo-methyl-benzyl carbinol reached by the direct method is identical with the one reached by Levene and Stevens by an indirect method which was based on the assumption that the cyclohexyl group has the same polarity as a normal hexyl group and that therefore levo-methylhexahydrobenzyl carbinol

$$\text{CH}_3\text{CHOHCH}_2\text{C}_6\text{H}_{11}$$
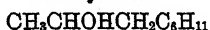
<div align="center">VII</div>

has the same configuration as levo-methylheptyl carbinol

$$\text{CH}_3\text{CHOHC}_7\text{H}_{15}$$

<div align="center">VIII</div>

The configuration of the levo-methylheptyl carbinol was determined by the rule of Levene and Haller.[9] The relationships are presented in the following set of figures.

$$
\begin{array}{ccccccc}
\text{CH}_2\text{OH} & & \text{CH}_2\text{C}_6\text{H}_5 & & \text{CH}_2\text{C}_6\text{H}_{11} & & \text{C}_7\text{H}_{15} \\
| & & | & & | & & | \\
\text{HCOH} & \rightarrow & \text{HCOH} & \rightarrow & \text{HCOH} & \rightarrow & \text{HCOH} \\
| & & | & & | & & | \\
\text{CH}_3 & & \text{CH}_3 & & \text{CH}_3 & & \text{CH}_3 \\
\text{Levo} & & \text{Levo} & & \text{Levo} & & \text{Levo}
\end{array}
$$
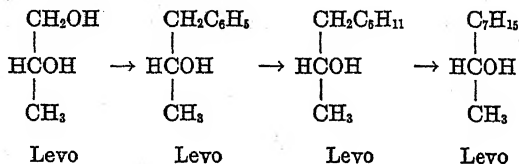
Thus the conclusion to be drawn from the observations thus far made is that the direction of rotation of a secondary carbinol is determined by the position of the heavier group, whether or not it contains a phenylic group as long as the latter is at a distance of

[9] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **79**, 478 (1928).

not less than 1 carbon atom from the asymmetric carbon atom. The effect of a phenylic group attached directly to the asymmetric carbon atom may be different.

Levo-Methylbutyl Carbinol from Dextro-Propylene Oxide.[10]

72 gm. (0.6 mol) of *n*-propylbromide were added to 14 gm. (0.6 mol) of magnesium and 300 cc. of anhydrous ether. When the reaction was completed, the flask was cooled in ice water. 34 gm. (0.6 mol) of optically active propylene oxide ($\alpha_D^{25} = +9.5°$), dissolved in 100 cc. of anhydrous ether, were then added in small portions. The reaction mixture was allowed to stand for 5 days. Most of the ether was then evaporated on a steam bath and the residue poured on ice. After the addition of dilute sulfuric acid the mixture was extracted with ether. The ether extracts were washed and dried over anhydrous potassium carbonate. The ether was removed by distillation and the residue fractionated. The fraction which boiled at 68–78° at 85 mm. had a rotation of $\alpha_D^{25} = +1.25°$. The fraction boiling at 85–95° (at 96 mm.) had a rotation of $\alpha_D^{25} = -0.35°$. All the fractions were united and converted into the phthalic acid ester which was purified and reconverted into the carbinol by the process of Levene and Mikeska.[11] From the crude product a fraction was obtained which boiled at 136–138° (corrected) at 754 mm. The substance had a composition which agreed with that of methylbutyl carbinol.

4.255 mg. substance: 10.970 mg. CO_2 and 5.200 mg. H_2O.
$C_6H_{14}O$ (102.14).   Calculated.   C 70.52, H 13.81
                    Found.   " 70.30, " 13.67

The substance had a rotation of $\alpha_D^{25} = -1.75°$ (without solvent).

α-Naphthylurethane of Methylbutyl Carbinol—To 1 gm. of the above substance were added 1.7 gm. of α-naphthyl isocyanate. The mixture was kept for a few minutes at 100°. On cooling a crystalline mixture was obtained. It was boiled with absolute alcohol and was filtered from the insoluble material. Water was

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[10] We wish to express our thanks to Mr. R. E. Marker for the preparation of some of the material.

[11] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **75**, 587 (1927).

added to the alcoholic filtrate until it became turbid.   On cooling, the urethane crystallized.   The substance was twice recrystallized from dilute alcohol.   It melted at 81–82.5° and analyzed as follows:

5.520 mg. substance: 0.266 cc. N (23°, 759 mm.).
$C_{11}H_{21}O_2N$.   Calculated.   N 5.16.   Found.   N 5.55

The specific rotation in absolute alcohol was as follows:

$$[\alpha]_D^{25} = \frac{-\ 0.24° \times 100}{1 \times 5.608} = -\ 4.28°$$

Levo-Methylbenzyl Carbinol from Dextro-Propylene Oxide

0.6 mol of optically active ($\alpha_D^{25} = +9.5°$) propylene oxide, dissolved in 100 cc. of anhydrous ether, was added gradually with stirring to a cooled solution of 0.6 mol of phenylmagnesium bromide in 250 cc. of ether.   The mixture was allowed to stand for 5 days at room temperature and then worked up in the same manner as described for methylbutyl carbinol.   32 gm. of a fraction were obtained which boiled at 105–106° at 15 mm.   It had the composition of methylbenzyl carbinol.

5.110 mg. substance: 14.945 mg. CO_2 and 3.860 mg. H_2O.
$C_9H_{12}O$ (136.14).   Calculated.   C 79.36, H 8.89
Found.   " 79.75, " 8.45

The rotation of the substance was $\alpha_D^{28} = -18.05°$ (homogeneous).

The above carbinol was purified through the phthalic acid ester sodium salt in the same manner as described for methylbutyl carbinol.   The phthalic acid ester was hydrolyzed by refluxing with a 10 per cent sodium hydroxide solution for 20 minutes.   The carbinol was then extracted with ether.   The ether solution was dried over anhydrous potassium carbonate, filtered, and fractionated.   A fraction was obtained which boiled at 114° at 25 mm. Its composition was that of methylbenzyl carbinol.

5.580 mg. substance: 16.275 mg. CO_2 and 4.305 mg. H_2O.
$C_9H_{12}O$ (136.14).   Calculated.   C 79.36, H 8.89
Found.   " 79.53, " 8.63

The rotation of the substance without solvent was $\alpha_D^{25} = -19.8°$.

α-Naphthylurethane of Levo-Methylbenzyl Carbinol

0.5 gm. of α-naphthyl isocyanate was added to 0.37 gm. of the above methylbenzyl carbinol. After thorough mixing of the substances, the material was kept for 15 minutes at 90°. The mixture then solidified. The urethane was crystallized from dilute alcohol. It melted at 111–113° and had the following composition.

4.850 mg. substance: 0.200 cc. N (23°, 760 mm.).
$C_{20}H_{19}O_2N$ (305.26). Calculated. N 4.59. Found. N 4.75

The specific rotation of the substance in absolute alcohol was as follows:

$$[\alpha]_D^{24} = \frac{-1.10° \times 100}{1 \times 3.476} = -31.6°$$

Methylbenzyl Ketone—In order to ascertain that the alcohol was methylbenzyl carbinol, the substance was oxidized to the corresponding ketone, and the latter was identified by its semicarbazone.

12 gm. of finely pulverized sodium dichromate were dissolved in 20 cc. of water. To this solution 10 gm. of methylbenzyl carbinol ($\alpha_D^{25} = -18.05°$) were added. To this mixture a solution of 15.0 gm. of sulfuric acid in 20 cc. of water was added with stirring, the operation lasting 3 hours. The mixture then was diluted and extracted with ether. The ether solution was washed, dried, and fractionated. The first fraction possessed an odor of benzaldehyde. The fractions which boiled between 108 and 115° at 26 mm. were united, taken up in ether, and neutralized with potassium carbonate. The ethereal solution was dried and the ether was removed by distillation. The residue was fractionated and the fraction boiling at 108° at 26 mm. was collected. The substance was neutral and was optically inactive. It had the composition of methylbenzyl ketone.

5.100 mg. substance: 15.000 mg. CO_2 and 3.195 mg. H_2O.
$C_9H_{10}O$ (134.12). Calculated. C 80.56, H 7.51
                              Found.   " 80.20, " 7.01

Semicarbazone of Methylbenzyl Ketone—1.3 gm. of the methylbenzyl ketone were added to a solution containing 1 gm. of potassium carbonate and 1.1 gm. of semicarbazide hydrochloride in 3.5

cc. of water.   A few cc. of methyl alcohol were added to the solution when the semicarbazone crystallized.   On cooling and washing with water and alcohol, 1.6 gm. of crude semicarbazone were obtained.   The substance was recrystallized from absolute alcohol. It had the following composition.

3.840 mg. substance: 0.754 cc. N (31°, 760.4 mm.).

$C_{10}H_{13}ON_3$ (91.18).   Calculated.   N 21.98.   Found.   N 22.05

It was melted at 188°.   The melting points recorded for this semicarbazone are 184–185°,[12] 188–189°,[13] 197°.[14]

[12] Wallach, O., *Ann. Chem.*, **332**, 317 (1904).

[13] Wolff, L., *Ann. Chem.*, **325**, 146 (1902).

[14] Tiffeneau, *Compt. rend. Acad.*, **142**, 1539 (1906).

ACETYL MONOSES

VI. THE RING STRUCTURE OF THE MANNOSE PENTACETATES

By P. A. LEVENE and R. STUART TIPSON

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Knowledge of the ring structure of the mannose pentacetates is of interest not only for its own sake but also for the reason that it may serve to elucidate the ring structures of the α- and β-mannoses. When the old form of mannose, having the specific rotation of $[\alpha]_D$ $-17°$, is acetylated in pyridine solution with acetic anhydride, a pentacetate[1] with a specific rotation of $[\alpha]_D$ $-24.9°$ is obtained; when the newer form of mannose[2] with the specific rotation $[\alpha]_D$ $+30°$ is acetylated in the same manner, the resulting product is a pentacetate[3] with the rotation of $[\alpha]_D$ $+57°$. It is generally known that in pyridine solution, mutarotation proceeds at a low rate and that when a given α- or β-isomer of a monose is acetylated in pyridine solution, the predominating form of the acetylated product has the same configuration of the carbon atom (1) as that of the parent substance. According to Hudson's nomenclature, the mannose with the specific rotation of $[\alpha]_D$ $-17°$ was considered the β form and the second form was regarded as the α form and the two pentacetates were regarded as the corresponding β and α forms. This view was generally accepted so long as one and the same ring structure was attributed to most simple sugars and their derivatives, and so long as the γ-sugars were regarded as rare and exceptional forms. In recent years, however, it has been shown that among sugar compounds many have the structure of the γ-derivatives, which, at the suggestion of Haworth, are now referred to as

[1] Fischer, E., and Oetker, R., *Ber. chem. Ges.*, **46**, 4034 (1913).

[2] Levene, P. A., *J. Biol. Chem.*, **57**, 329 (1923).

[3] Levene, P. A., *J. Biol. Chem.*, **59**, 141 (1924).

furanoses to indicate their 5-membered ring structure.   In the opinion of Hudson, some of the γ forms may have ring structures of still smaller size.   In the special case of mannose, Hudson[4] recently attributed different ring structures to α-d-mannose ($[\alpha]_D$ $+30°$) and β-d-mannose ($[\alpha]_D$ $-17°$).   If this assumption of Hudson is correct, then the two known forms of pentacetates of d-mannose also should possess different ring structures.   On the other hand, if it were shown that the two pentacetates had an identical ring structure, apparently the only conclusion possible would be that the two mannoses are the α and β forms of the identical ring isomer.   In view of these considerations, it seemed desirable to determine experimentally the ring structures of the two d-mannose pentacetates.

Bergmann and Freudenberg[5] have demonstrated that glucal contains the pyran ring; we also know that bromotetracetyl glucose leads to the common tetracetyl methylglucoside containing a 6-membered ring.   From these two facts it is concluded that the transformation of a bromotetracetyl monose into the corresponding glucal[6] does not involve a ring shift.   Hence, if it were possible to transform 1-bromotetracetyl-d-mannose into triacetyl glucal, then the ring structure of the bromotetracetate of d-mannose and with it that of the parent pentacetate would be elucidated.   This reaction was actually accomplished, the starting material being a pure crystalline form of the 1-bromotetracetyl-d-mannose, derived from the β-d-mannose pentacetate and the product was found to be ordinary triacetyl glucal.   Hence it is warranted to attribute to the β-d-mannose pentacetate a 6-membered ring structure.   In addition, we now find that when the same bromotetracetyl mannose is acted upon by silver acetate, a pentacetate is formed which is identical with the α-d-mannose pentacetate and, on the other hand, that the acetylation of the tetracetyl mannose obtained from the 1-bromotetracetyl-d-mannose leads to the β-pentacetate of mannose.   There would thus appear to be little doubt that the two pentacetates are the α and β forms of the same

[4] Hudson, C. S., *J. Am. Chem. Soc.*, **52**, 1680 (1930).

[5] Bergmann, M., and Freudenberg, W., *Ber. chem. Ges.*, **62**, 2783 (1929).

[6] Glucal in this case is employed in a generic sense, in a manner similar to the use of the term glucoside.

ring isomer, the relationship of the two forms being represented as follows:

β-d-mannose pentacetate → bromotetracetyl-d-mannose → α-d-mannose pentacet:

d-mannose tetracetate
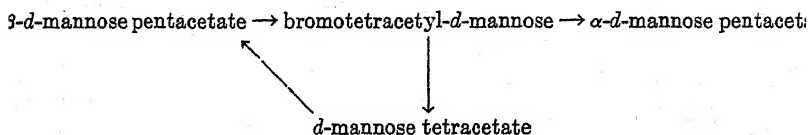
This conclusion, in its turn, indicates that the two mannoses also are the α and β forms of the same ring isomer. This view is in harmony with the fact that the d-mannoses with $[\alpha]_D$ +30° and $[\alpha]_D$ −17° display in aqueous solution velocities of mutarotation of the same order of magnitude, whereas the third form, discovered by Dale[7] and regarded by Hudson as the β form of the ordinary α-d-mannose, mutarotates with a velocity of an entirely different order of magnitude.

Incidentally, at this time we may record the fact that dry sodium methylate acting on crystalline 1-bromotetracetyl mannose in toluene solution forms the peculiar tetracetyl methylmannoside to which Freudenberg[8] and Braun[9] recently attributed the structure:

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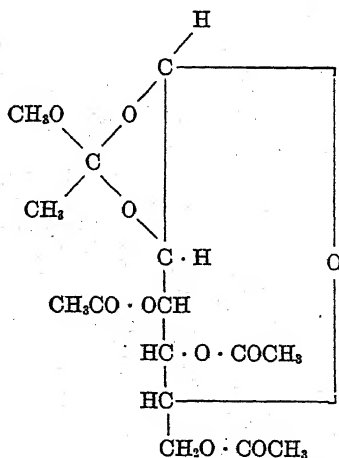
[7] Dale, J. K., *J. Am. Chem. Soc.*, **51**, 2788 (1929).

[8] Freudenberg, K., *Naturwissenschaften*, **18**, 393 (1930).

[9] Braun, E., *Naturwissenschaften*, **18**, 393 (1930).

The substance is generally prepared by the action on 1-bromo-tetracetyl-*d*-mannose of methyl alcohol in the presence of silver carbonate, a reaction supposedly much more complex than the direct action of dry sodium methylate. Hence it was expected that, in the latter case, an ordinary tetracetyl methylmannoside would be obtained.

We take this occasion to point out another conclusion reached by Hudson which is in disagreement with the results of observations made in this laboratory and reported several years ago. It concerns the ring structure of the α-*d*-methylmannoside. Hudson[4] assigns to this glucoside the <1,4> lactal structure, whereas to its tetramethyl derivative he attributes the <1,5> lactal structure, thus assuming a ring-shift in course of methylation of the α-methylmannoside. The <1,5> lactal structure of normal methylmannoside was first demonstrated by Levene and Meyer[10] in 1924, although the substance had been methylated by Irvine and Moodie[11] in 1905. The tetramethylmannose prepared from this mannoside was shown by Levene and Meyer to yield on oxidation a tetramethyl mannonolactone distinctly different from that formed on methylation of that mannonic lactone which is known to possess the <1,4> lactone structure. The differences in the lactones were later substantiated by Levene and Simms[12] on the basis of the rates of lactone formation of the two corresponding acids. Later Goodyear and Haworth[13] arrived at the same conclusion by a method of oxidative degradation. Levene and Meyer[14] have also furnished evidence tending to show that this ring structure of the final product is not the result of a shift in the ring of a methylfuranoside. A γ-methylmannoside had been prepared by Irvine and Burt[15] in 1924. From this mannoside Levene and Meyer prepared a lactone identical with that obtained on methylation of the γ-mannonic lactone. Levene and Meyer then concluded that the syrupy-γ-mannoside of Irvine and Burt "at least in part consists of a <1,4> mannoside." Recently

[10] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **60**, 167 (1924).

[11] Irvine, J. C., and Moodie, A. M., *J. Chem. Soc.*, **87**, 1462 (1905).

[12] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, **65**, 31 (1925).

[13] Goodyear, E. H., and Haworth, W. N., *J. Chem. Soc.*, 3136 (1927).

[14] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **76**, 809 (1928).

[15] Irvine, J. C., and Burt, W., *J. Chem. Soc.*, **125**, 1343 (1924).

Haworth and coworkers[16] obtained the γ-methylmannoside in crystalline form and showed that this mannoside on methylation with subsequent hydrolysis and oxidation yielded the γ-lactone previously described by Levene and Meyer. By means of degradative oxidation they have demonstrated[13] the presence in this lactone of a 5-membered ring. Thus, Haworth has likewise shown that the methylation of methylmannofuranoside is accomplished without a ring shift. The trustworthiness of this mode of reasoning was recently demonstrated by Haworth[17] by a method not involving methylation.

It therefore seems that in the case of mannose pentacetates as well as in the case of methylmannosides, the conclusions reached by the chemical methods are in disagreement with those arrived at by Hudson's method of "isorotation" calculations.

<div align="center">EXPERIMENTAL</div>

Preparation of Crystalline Bromotetracetyl Mannose—50 gm. of finely powdered β-mannose pentacetate were mixed with 200 cc. of glacial acetic acid containing 40 per cent of dry hydrogen bromide. The resulting solution was allowed to stand at room temperature for 60 minutes, after which the hydrogen bromide gas was removed under diminished pressure at room temperature. The solution was then diluted with 600 cc. of toluene and evaporated to a thick syrup under diminished pressure at 35°. Two further portions of 200 cc. of toluene were run in and evaporated off. This syrup was now dissolved in 200 cc. of benzene and the solution evaporated to a thick syrup. Traces of solvent were removed at high vacuum at 40°.

The resulting thick, very pale yellow, syrup was dissolved in the minimum of cold, dry ether, a further 20 cc. of ether were added, and then petroleum ether was added to incipient turbidity. A little charcoal was added, the mixture shaken, and filtered on a fluted filter, the filtrate obtained being absolutely colorless.

On nucleation of the filtrate with an authentic crystal of bromotetracetyl mannose and allowing to stand in the refrigerator, crystallization rapidly set in. Yield, 32 gm. of colorless crystals; m.p., 53–54°.

[16] Haworth, W. N., Hirst, E. L., and Webb, J. I., *J. Chem. Soc.*, 651(1930).
[17] Haworth, W. N., *J. Am. Chem. Soc.*, **52**, 4168 (1930).

Its rotation was

$$[\alpha]_D^{25} = \frac{+\ 3.00° \times 100}{2 \times 1.218} = +\ 123.2° \text{ (in chloroform)}$$

Preparation of Triacetyl Glucal from Bromoacetyl Mannose—30 gm. of zinc dust were added to 250 cc. of cold 50 per cent aqueous acetic acid with vigorous mechanical stirring at 0°. Then 25 gm. of finely powdered, crystalline bromoacetyl mannose dissolved in 50 cc. of glacial acetic acid were quickly added (in one portion) and the mixture was stirred for some 90 minutes at 0°. The reaction was followed by quantitative estimation of the bromine absorption of 1 cc. test portions after various time intervals. When no further increase in unsaturation occurred, the stirring was stopped and the mixture filtered.

The filtrate was extracted fifteen times with 100 cc. portions of toluene, after which the aqueous layer had lost its power of decolorizing very dilute bromine water. The toluene extract was evaporated to a thick syrup under diminished pressure at 40°. Two further portions of 200 cc. of toluene were run in and evaporated off. Finally, this syrup was dissolved in 200 cc. of benzene and evaporated to a thick syrup which usually crystallized at this stage.

This product was dissolved in the minimum of ether and nucleated with an authentic specimen of triacetyl glucal prepared from bromotetracetyl glucose. On cooling in ice and scratching, it rapidly set to a solid mass of crystals. After one recrystallization from ether and one from a mixture of absolute alcohol and petroleum ether, the product had a m.p. of 54–55°; yield, 12 gm. of triacetyl glucal. It had the following composition.

5.695 mg. substance: 11.055 mg. CO_2 and 3.125 mg. H_2O.
$C_{12}H_{16}O_7$.   Calculated.   C 52.92, H 5.9
                 Found.   " 52.93, " 6.1

Its rotation was

$$[\alpha]_D^{25} = \frac{-\ 0.66° \times 100}{2 \times 2.025} = -\ 16.3° \text{ (in absolute ethyl alcohol)}$$

Action of Silver Acetate upon Bromoacetyl Mannose—To a solution of 17 gm. of crystalline bromoacetyl mannose in 90 cc. of

toluene were added 9 gm. of dry, finely powdered silver acetate and the mixture was heated under reflux at 95° for 20 minutes. It was then shaken with a little charcoal, filtered, and the filtrate (having been found free of bromine) evaporated under diminished pressure at 35° to a very thick syrup. Final traces of solvent were removed at the high vacuum pump at 40°. Yield, 15 gm.

Test samples of the substance, dissolved in 95 per cent ethyl alcohol were nucleated with the α and β forms of pentacetyl mannose respectively, but crystallization could not be induced.

The rotation of the syrupy substance was

$$[\alpha]_D^{23} = \frac{+ 0.48° \times 100}{1 \times 1.140} = + 42.1° \text{ (in chloroform)}$$

The analysis of this thick syrup was as follows:

3.855 mg. substance: 6.990 mg. CO_2 and 2.080 mg. H_2O.
$C_{16}H_{22}O_{11}$. Calculated. C 49.20, H 5.7
Found.   " 49.45, " 6.0

As crystallization of this crude product could not be induced, it was subjected to distillation at 190° at a pressure of 0.01 mm. (compare Levene[3]). The distillate, on cooling to room temperature, set to a hard glass, very pale yellow in color. On being moistened with 1 drop of absolute alcohol and left to stand at room temperature overnight, it set to a solid mass of crystals having a m.p. of 64° and a rotation of

$$[\alpha]_D^{26} = \frac{+ 1.54° \times 100}{2 \times 1.361} = + 56.6° \text{ (in chloroform)}$$

After recrystallization from very dilute aqueous alcohol it had a m.p. of 74°.

Its analysis was as follows:

5.275 mg. substance: 9.525 mg. CO_2 and 2.765 mg. H_2O.
$C_{16}H_{22}O_{11}$. Calculated. C 49.20, H 5.7
Found.   " 49.24, " 5.9
100 mg. substance required for Sample A 12.67, and Sample B, 12.82 cc. 0.1 N NaOH (alkaline hydrolysis).
$C_6H_7O_6 \cdot (COCH_3)_5$. Calculated. $COCH_3$ 55.1
Found. Sample A.   "   54.5
"   B.   "   55.1

100 mg. substance required for Sample A 12.85, and Sample B, 12.83 cc. 0.1 N NaOH (control with authentic β-pentacetyl mannose).
<div align="center">
Found.   Sample A.   COCH₃ 55.3
"   B.   "   55.2
</div>

In the meantime a portion which had not been subjected to distillation at high vacuum crystallized spontaneously, the product having properties identical with those recorded above, thus indicating that the distillation had no deleterious effect.

Action of Sodium Methoxide upon Bromoacetyl Mannose—2 gm. of sodium were dissolved in 200 cc. of methyl alcohol (dry and freshly distilled from sodium). 56 cc. of this solution were evaporated to dryness and the product shaken with dry ether and the ether quickly decanted. The white powder was then twice washed by decantation with toluene (dried and freshly distilled) and traces of solvent removed under diminished pressure.

A solution of 10 gm. of crystalline bromoacetyl mannose in 50 cc. of dry toluene was now added, and the mixture allowed to stand 16 hours at room temperature. A small test portion was then filtered and the filtrate found to contain much bromine.

After heating under reflux for 3 to 4 hours in a glycerol bath at 110° and allowing to stand for 48 hours at room temperature, a filtered test portion was found to contain no bromine. The main product was therefore filtered and the filtrate evaporated under diminished pressure to a thick syrup, which, after traces of solvent had been removed at the Hyvac pump, soon crystallized spontaneously to a solid mass. Yield, 8.4 gm. It was dissolved in dry ether, shaken with a little charcoal, filtered, and the filtrate evaporated. The product was recrystallized from dry methyl alcohol, m.p. 104–105°. It had the following composition.

4.270 mg. substance: 7.830 mg. CO_2 and 2.425 mg. H_2O.
5.455   "   "   : 3.170 "   AgI.
<div align="center">
$C_{15}H_{22}O_{10}$.   Calculated.   C 49.70, H 6.1, OMe 8.56
Found.   " 50.00, " 6.3, "   7.68
</div>

Its rotation was

$$[\alpha]_D^{n} = \frac{-\ 0.60° \times 100}{2 \times 1.326} = -\ 22.6° \text{ (in chloroform)}$$

100 mg. substance required 8.48 cc. 0.1 N NaOH (alkaline hydrolysis).
$C_9H_{13}O_7 \cdot (CH_3CO)_3$.   Calculated. $COCH_3$ 35.65 (for 3 hydrolyzable
acetyl groups)

Found.   "   36.48

Preparation of 2,3,4,6-Tetracetyl Mannose—10 gm. of finely
powdered crystalline bromoacetyl mannose were dissolved in 50 cc.
of dry ether and 5 gm. of dry silver carbonate added.   To the
suspension was added, drop by drop, 0.5 cc. of distilled water with
vigorous shaking.   The reaction was followed by removing a small
test portion from time to time, filtering, and shaking the filtrate
with silver nitrate solution.   After 2 hours the reaction was com-
plete, as indicated by the absence of bromine in the filtrate, but
no crystals had separated (although in parallel experiments with
bromoacetyl glucose the tetracetyl glucose crystallizes out towards
the end of the operation).   The main reaction mixture was now
filtered, the silver salts washed repeatedly with dry ether, the com-
bined filtrate shaken with a little charcoal, filtered, and the filtrate
evaporated under diminished pressure to a thick colorless syrup.
This was dissolved in a small quantity of dry ether, petroleum
ether added to incipient turbidity, and, on cooling and scratching,
tetracetyl mannose rapidly crystallized.   Yield, 8 gm.

Unlike tetracetyl glucose, the product is very soluble in ether
and is best recrystallized from a mixture of ether and petroleum
ether, whereby a product is obtained of m.p. 93°, strongly re-
ducing towards boiling Fehling's solution.

$$[\alpha]_D^{27} = \frac{+ 0.53° \times 100}{2 \times 1.006} = + 26.3° \text{ (in chloroform)}$$

It had the following composition.

4.530 mg. substance: 8.045 mg. CO_2 and 2.365 mg. H_2O.
$C_{14}H_{20}O_{10}$.   Calculated.   C 48.25, H 5.8
Found.   " 48.44, " 5.8

It therefore differs from the (?) 2,3,4,6-tetracetyl mannose de-
scribed by Micheel and Micheel[18] which had a m.p. of 159–160°,
$[\alpha]_D^{19} = -24.2°$ (in chloroform).

Acetylation of Tetracetyl Mannose—1.5 gm. of crystalline tetra-

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[18] Micheel, F., and Micheel, H., *Ber. chem. Ges.*, **63**, 386 (1930).

cetyl mannose were dissolved in a cold mixture of 10 cc. of pyridine and 10 cc. of acetic anhydride and the resulting solution was kept at 0° for 50 hours.

The reaction mixture was diluted with 200 cc. of chloroform and the product isolated (by washing successively with ice-cold diluted sulfuric acid, ice-cold dilute sodium bicarbonate solution, and finally with ice water until neutral, drying over anhydrous sodium sulfate, and evaporating to a thick syrup). The syrup obtained crystallized spontaneously on cooling. On recrystallization from 95 per cent ethyl alcohol it had a m.p. of 117°; yield, 1 gm.

Its rotation was

$$[\alpha]_D^n = \frac{-0.52° \times 100}{1 \times 2.162} = -24.1° \text{ (in chloroform)}$$

The substance had the following composition.

4.995 mg. substance: 8.960 mg. CO_2 and 2.500 mg. H_2O.
$C_{16}H_{22}O_{11}$.   Calculated.   C 49.20, H 5.7
Found.   " 48.92, " 5.6

THE EFFECTS OF VITAMIN DEFICIENCY UPON THE COEFFICIENTS OF DIGESTIBILITY OF PROTEIN, FAT, AND CARBOHYDRATE

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The profound metabolic changes which are brought about by a vitamin-deficient diet suggest that the digestive processes may in some way be affected by the lack of these accessory substances. It has been suggested that vitamins may affect the glandular secretory processes connected with digestion or that they may influence the absorption of foods from the alimentary tract and their subsequent oxidation. If the digestive process is affected by a lack of vitamins, we should expect such a disturbance to be evidenced by an inefficient utilization of food.

There has been no work done upon the effect of vitamin A on the utilization of food except in so far as a vitamin A deficiency is included in conditions of polyavitaminosis. Bickel (1) found that although both fat and protein absorption and oxidation are quantitatively elevated during avitaminosis, they are, for the most part, qualitatively normal. Bickel (2) further shows that although during complete avitaminosis the body weight of guinea pigs diminishes and anemia and atrophia changes become prominent, the diet is still quantitatively absorbed. He is of the opinion that vitamins do not influence either the digestion or absorption of food materials. He believes that in the absence of vitamins the cells gradually lose their ability to resynthesize and store the food digestion products in the blood and consequently the animal must sacrifice its own tissue.

According to Never (3), the secretory activity and acidity of the gastric juice are not abnormal during avitaminosis; but as the condition progresses, the activity of the pepsin increases and the digestive glands tend to accumulate pepsin and trypsin or their precursors. He further reports that there is a disturbance in absorption and secretion during avitaminosis and that the nitrogen balance is disturbed.

Lavrov and Matzko (4) found that when starvation was prevented in chickens during a B avitaminosis, the utilization of the food nitrogen

remained satisfactory but that there was a negative nitrogen balance and an increased uric acid excretion which shows that in the initial stages of avitaminosis there is an intense destruction of nitrogenous materials.

Ederer (5) found that vitamin B has a profound influence on protein and carbohydrate assimilation on diets made up of one or of the other of these foodstuffs. Karr (6) found that nitrogen utilization in the digestive tract is unaffected by the lack of the vitamin B complex. In a metabolic study of scurvy, Baumann and Howard (7) report a high negative nitrogen balance during the scorbutic period. Shipp and Zilva (8) explain this as due entirely to an insufficient intake of nitrogen and calories. To overcome this difficulty, Jarussowa (9) forcibly fed animals on a scorbutic diet, so that they maintained their weight for a period of 24 days, during which time they were in positive nitrogen balance. Immediately thereafter the nitrogen balance became negative. She concludes that during the development of scurvy, the nitrogen balance changes from positive to negative.

Shipp and Zilva, in their study of the effect of vitamin C deficiency upon the metabolism of growing guinea pigs, found no indications of a disturbed absorption or retention of nitrogen during the early stages of the development of scurvy. The nitrogen balance became negative only when the intake of food was diminished as a consequence of the disease.

There seems to have been no work done upon the effect of vitamin D upon the digestibility of foods, except as it is included in the investigations by Bickel of metabolism during polyavitaminosis.

The purpose of the present work was to study the individual effects of vitamins A, B_1, B_2, C, and D upon the utilization of foods by determining the coefficients of digestibility of protein, fat, and carbohydrate in animals deprived of the particular vitamin under consideration and in those receiving a complete ration. The term digestibility as used here should be considered as the apparent rather than the real digestibility.

Method

The general plan of the experiment was to keep the animals upon a diet deficient in one of the vitamins until the reserve of that vitamin stored in the animal was entirely depleted. The missing supplement was then added to the diet of part of the animals while their litter mates were continued as controls on the deficient ration. Upon the depletion of the stored vitamin the rats were placed in metabolism cages so that the urine and feces might be collected separately. To avoid a possible misinterpretation of the

results, due to a difference in food consumption in the two groups, the food intake of the animals on the complete diet was in most cases limited to that of the animals on the deficient diet.

The diets were analyzed for protein, fat, moisture, ash, and fiber. The nitrogen of the urine and feces was determined by the Kjeldahl-Gunning method. The fat, crude fiber, moisture, and ash of the feces and diet were determined by the Official Method of Analysis of the Association of Official Agricultural Chemists. The nitrogen balance was determined and the coefficients of digestibility of proteins, fats, and carbohydrates were calculated as follows:

$$\text{Coefficient of digestibility} = \frac{\text{amount ingested} - \text{amount in feces}}{\text{amount ingested}}$$

EXPERIMENTAL

Vitamin A

Twelve rats were evenly distributed in four cages so that the rats in Cages 1 and 3 were litter mates and controls, respectively, of those in Cages 2 and 4. The rats were kept on screens to prevent coprophagy. They were fed the following vitamin A-free irradiated ration.

	per cent
Casein (alcohol-extracted)	18
Yeast	8
Dextrin	56
Salt mixture*	3
Crisco	15

* McCollum's Salt Mixture 185 (McCollum, E. V., and Simmonds, N., J. Biol. Chem., **33**, 63 (1918)).

When losses in weight indicated that the stored vitamin A was exhausted, the rats were transferred to metabolism cages and the collection of urine and feces was begun. Each rat in Cages 2 and 4 was given 0.5 cc. of cod liver oil daily. The animals in Cages 1 and 3 were continued on the deficient ration. The food intake of the rats in Cages 2 and 4 was limited to that of their litter mates in Cages 1 and 3, respectively. However, one of the rats in Cage 3 died, so an allowance was made for this in the food intake of the rats of Cage 4.

The feces and urine were collected in three periods of 3 days each. The feces were dried at 100° immediately upon collection and the urine was preserved with acid.

Table I shows the amount of nitrogen consumed by the animals of each cage during each period of the experiment and the amount of nitrogen occurring in the urine and feces. The nitrogen balance and the coefficients of digestibility of protein, fat, and carbohydrate are calculated.

TABLE I

Nitrogen Balance and Coefficients of Digestibility of Protein, Fat, and Carbohydrate in Rats with and without Vitamin A

Experiment I.

Diet	Cage No.	Period	Nitrogen						Fat coefficient	Carbohydrate coefficient
			Intake per period	Output in			Balance	Coefficient		
				Urine	Feces	Total				
			gm.	*gm.*	*gm.*	*gm.*	*gm.*			
No vitamin A........	1	1	1.26	0.77	0.18	0.95	+0.31	85	97	98
" " "		2	1.05	0.81	0.18	0.99	+0.06	83	96	99
		3	1.26	0.76	0.19	0.95	+0.32	83	99	98
Vitamin A..........	2	1	1.26	0.76	0.17	0.93	+0.33	86	98	99
" "		2	1.05	0.70	0.11	0.81	+0.24	90	98	98
" "		3	1.26	0.70	0.29	0.99	+0.28	77	95	98
No vitamin A........	3	1	1.08	0.86	0.22	1.08	+0.01	82	96	97
" " "		2	0.86	0.64	0.17	0.81	+0.05	80	97	98
" " "		3	1.05	0.59	0.20	0.80	+0.25	81	97	97
Vitamin A..........	4	1	1.39	0.68	0.19	0.87	+0.52	88	96	97
" "		2	1.02	0.65	0.22	0.87	+0.14	78	96	98
" "		3	1.03	0.91	0.16	1.07	−0.03	85	98	99

It will be observed from Table I that the animals on the vitamin A-deficient diet and those on the complete ration were in positive nitrogen balance except during one period when there was a slight negative balance occurring in one cage of rats on a complete diet, due to the low food intake.

The experiment was repeated with twelve animals as described above, with the exception that collections were made for three periods of 4 days each. A positive nitrogen balance was found in

all the cages for all periods except for one period in one cage of rats which received vitamin A.   The rats which received no vitamin A showed the following average coefficients of digestibility: for protein, 82; for fat, 97; and for carbohydrate, 96.   For those animals which received vitamin A the average coefficients of digestibility were: 79 for protein, 98 for fat, and 93 for carbohydrate.

The average coefficients of digestibility obtained in the two experiments are as follows:

	No vitamin A	Vitamin A
Protein..	83	83
Fat...	97	92
Carbohydrate.................................	97	96

Vitamin A appears to have no effect upon the digestibility of food.

Vitamins B_1 and B_2

Twelve young rats were evenly distributed in four cages so that each rat had a litter mate in each of the other three cages. They received the following vitamin B-free ration.

<div align="right">per cent</div>

Casein (water-washed)..................................... 18
Dextrin... 60
Salt mixture*.. 3.5
Cod liver oil.. 3.5
Crisco.. 15

* McCollum's Salt Mixture 185.

Upon the depletion of the stored vitamin, as evidenced by a loss in body weight, metabolic studies were begun.   The rats of Cage 1, serving as controls, were continued upon the deficient ration without the addition of a supplement.   Each rat in Cage 2 was given 0.4 cc. of an alcoholic extract of rice polishings daily; in Cage 3 each animal received 0.75 gm. of autoclaved yeast; and in Cage 4, each received 0.75 gm. of whole yeast daily.   The rats were allowed to eat the basal diet *ad libitum*.   Urine and feces were collected for six periods of 4 days each.

Table II presents the nitrogen balance and the coefficients of digestibility as determined in this experiment.

TABLE II

Nitrogen Balance and Coefficients of Digestibility of Protein, Fat, and Carbohydrate in Rats with and without Vitamin B Complex

Experiment I.

Diet	Cage No.	Period	Nitrogen Intake per period	Output in Urine	Feces	Total	Balance	Co-efficient	Fat coefficient	Carbohydrate coefficient
			gm.	*gm.*	*gm.*	*gm.*	*gm.*			
No vitamin B.......	1	1	0.88	0.54	0.15	0.69	+0.19	83	97	91
" " "		2	0.74	0.44	0.07	0.51	+0.23	91	96	92
" " "		3	0.67	0.55	0.03	0.58	+0.09	96		
" " "		4	0.54	0.54	0.10	0.64	−0.10	81	94	94
" " "		5	0.54	0.46	0.05	0.51	+0.04	92	97	95
" " "		6	0.47	0.61	0.04	0.65	−0.18	91	90	88
Vitamin B..........	2	1	1.12	0.61	0.14	0.75	+0.37	87	98	95
" "		2	0.80	0.49	0.06	0.55	+0.26	93	99	98
" "		3	0.78	0.45	0.09	0.54	+0.24	89	98	97
" "		4	0.89	0.50	0.09	0.59	+0.29	87	98	97
" "		5	0.94	0.59	0.07	0.66	+0.28	92	97	97
" "		6	1.00	0.60	0.07	0.67	+0.33	93	97	95
Vitamin B_2..........	3	1	1.62	0.80	0.42	1.22	+0.40	74	94	91
" "		2	1.38	0.69	0.26	0.95	+0.42	81	95	94
" "		3	1.18	0.63	0.29	0.92	+0.26	76	94	92
" "		4	1.21	0.65	0.28	0.93	+0.28	77	94	94
" "		5	1.56	0.66	0.29	0.95	+0.61	81	92	97
" "		6	1.43	0.94	0.35	1.29	+0.15	76	96	99
Vitamin B_1 + B_2....	4	1	1.80	1.00	0.26	1.26	+0.53	85	90	90
" " " "		2	1.48	0.73	0.14	0.87	+0.61	90	94	92
" " " "		3	1.46	0.82	0.15	0.97	+0.49	90	95	97
" " " "		4	1.88	0.86	0.25	1.11	+0.78	87	91	90
" " " "		5	1.88	1.04	0.16	1.20	+0.68	92	94	93
" " " "		6	1.90	1.10	0.22	1.32	+0.58	88	95	90

It will be observed from Table II that in this experiment during which there was no control of the food intakes, the animals of the control cage showed a slight positive nitrogen balance throughout

the four periods and a negative balance for two periods.   The rats receiving either vitamin B_1 or B_2 showed an increased positive balance over that of the controls, while those animals which were given whole yeast maintained a positive balance noticeably larger than that shown in the other cages.

A similar metabolic study was made on sixteen rats.   In this experiment, vitamin B_1 was supplied by 40 mg. of activated fullers' earth per rat per day.   The food intake of the animals of all cages was limited to that of the rats which received no vitamin B. Collections of urine and feces were made for four periods of 3 days each.   In this experiment the rats of all cages maintained a slight positive nitrogen balance.   The average coefficients obtained in the two experiments are summarized in Table III.

TABLE III

Average Coefficients of Digestibility

	Control	Vitamin B_1	Vitamin B_2	Vitamins B_1 and B_2
Experiment I				
Protein.........................	89	84	78	89
Fat.............................	96	97	94	93
Carbohydrate...................	92	97	94	92
Experiment II				
Protein.........................	84	83	77	81
Fat.............................	96	97	96	95
Carbohydrate...................	97	97	94	95

In both experiments the coefficient of digestibility of protein was noticeably lower in those animals which received vitamin B_2 alone than in the controls or in those rats which received vitamin B_1 or both vitamins B_1 and B_2.   With this exception, the presence of vitamin B_1, B_2, or both B_1 and B_2, does not seem to affect the coefficients of digestibility of protein, fat, and carbohydrate.

Vitamin C

In the first experiment, three young guinea pigs were used. They were kept on screens and were given the following vitamin C-free diet.

Ground oats... 59
Skimmed milk powder, heated for 2 hrs. at 110°.............. 30
Butter fat.. 10
Sodium chloride... 1

Within 15 days all of the animals had lost weight and were clearly suffering from scurvy. At this point collection of the urine and feces was begun. The guinea pigs were kept in me-

TABLE IV

Nitrogen Balance and Coefficients of Digestibility of Protein, Fat, and Carbohydrate in Guinea Pigs with and without Vitamin C

Experiment I.

Diet	Cage No.	Period	Nitrogen						Fat coefficient	Carbohydrate coefficient
			Intake per period	Output in			Balance	Coefficient		
				Urine	Feces	Total				
		days	gm.	gm.	gm.	gm.	gm.			
No vitamin C........	1	3	1.79	0.35	0.83	1.18	+0.61	54	86	63
" " "		3	1.20	0.37	0.69	1.06	+0.14	42	85	54
" " "		3	0.65	0.36	0.52	0.88	−0.23	21	73	56
" " "		3	0.02	0.45	0.07	0.52	−0.50			
Vitamin C...........	2	3	1.75	0.34	1.03	1.37	+0.38	41	77	56
" "		3	1.29	0.35	0.75	1.10	+0.18	42	70	54
" "		3	0.67	0.33	0.43	0.76	−0.09	36	83	47
" "		3	1.47	0.36	0.72	1.08	+0.39	51	86	48
Vitamin C..........	3	3	1.77	0.42	1.09	1.51	+0.26	38	81	52
" "		3	1.28	0.37	0.73	1.10	+0.19	43	81	57
" "		3	0.85	0.39	0.23	0.63	+0.22	73	93	71
" "		3	1.67	0.41	1.20	1.60	+0.06	28	76	54

tabolism cages arranged with a chute into which the animal had to go to reach its food. This arrangement was used to prevent scattering the food over the feces. One of the guinea pigs was continued on the deficient diet in order to serve as a control; each of the remaining animals was given 5 cc. of tomato juice daily. The food consumption of the animals receiving the supplement was limited to that of the control for the first two periods

of 3 days each.   During the remaining two periods they were allowed to eat the diet *ad libitum.*

Table IV shows the nitrogen balance and the coefficients of digestibility obtained in the experiment.

In the second experiment in which two guinea pigs were used, the urine and feces were collected from the time that the animals were placed on the deficient diet.   As soon as scurvy developed each guinea pig was given 10 cc. of tomato juice daily.   Collections were made on Guinea Pig 4 for 43 days and on Guinea Pig 5 for 20 days, at the end of which time it died.   The average coefficients of digestibility determined for the periods during which the guinea pigs received no vitamin C were as follows: protein, 50; fat, 90; and carbohydrate, 66.   For the periods during which the guinea pigs received vitamin C the following coefficients were found: protein, 49; fat, 89; and carbohydrate, 70.   Guinea Pig 4 maintained a positive nitrogen balance except during the period immediately following the introduction of vitamin C into the diet.   Guinea Pig 5 was in negative balance in all periods except one.

The average coefficients of digestibility observed in the two experiments on the five animals are as follows:

	No vitamin C	Vitamin C
Protein	46	46
Fat	86	85
Carbohydrate	63	62

The low coefficients of digestibility of protein were apparently not due to a lack of vitamin C, for they remained low even after the administration of the tomato juice.   The digestibility of foods does not appear to be affected by vitamin C.

Vitamin D

Twelve rats were distributed among four cages so that the rats in Cages 1 and 3 were litter mates respectively, of those in Cages 2 and 4.   The rats were kept in the dark and were placed on raised screens to prevent coprophagy.   They were fed the following vitamin D-free ration.

per cent

Yellow corn... 76
Wheat gluten.. 20
Calcium carbonate.. 3
Sodium chloride.. 1

The rats were placed in metabolism cages as soon as x-ray examination of the bones of the right hind leg showed the animals

TABLE V

Nitrogen Balance and Coefficients of Digestibility of Protein, Fat, and Carbohydrate in Rats with and without Vitamin D

Experiment I.

Diet	Cage No.	Period	Nitrogen						Fat coefficient	Carbohydrate coefficient
			Intake per period	Output in			Balance	Coefficient		
				Urine	Feces	Total				
			gm.	*gm.*	*gm.*	*gm.*	*gm.*			
No vitamin D.......	1	1	1.38	1.00	0.18	1.18	+0.20	89	84	93
" " "		2	1.56	1.10	0.20	1.30	+0.26	87	84	93
" " "		3	1.61	1.01	0.23	1.24	+0.36	85	84	92
" " "		4	1.45	1.03	0.25	1.29	+0.17	83	84	93
Vitamin D	2	1	1.32	1.08	0.17	1.25	+0.10	87	84	92
" "		2	1.56	1.10	0.21	1.31	+0.25	86	83	92
" "		3	1.51	1.10	0.18	1.26	+0.25	88	85	93
" "		4	1.26	1.04	0.19	1.23	+0.03	87	84	92
No vitamin D.......	3	1	1.49	0.97	0.17	1.14	+0.35	88	87	92
" " "		2	1.70	1.17	0.19	1.36	+0.34	89	89	93
" " "		3	1.71	1.25	0.20	1.45	+0.26	88	85	92
" " "		4	1.71	1.02	0.23	1.25	+0.46	87	85	92
Vitamin D..........	4	1	1.43	1.02	0.14	1.17	+0.26	90	88	93
" "		2	1.52	0.97	0.16	1.13	+0.39	87	86	92
" "		3	1.40	1.03	0.14	1.17	+0.23	90	88	93
" "		4	1.53	1.37	0.15	1.52	+0.01	90	89	93

to be rachitic. The controls in Cages 1 and 3 were continued on the vitamin D-deficient diet while those in Cages 2 and 4 were given the basal diet which had been irradiated. The food intake of the rats in Cages 2 and 4 was limited to that of their litter mates in Cages 1 and 3, respectively. The feces and urine were

collected in four periods of 3 days each.   x-Ray pictures at the end of the experiment indicated that the animals had been cured.

In Table V are found the nitrogen balances and coefficients of digestibility as determined in this experiment.

This experiment was repeated with twelve rats, as described above. The urine and feces were collected in four periods of 3 days each. In this experiment, cod liver oil was the source of vitamin D. Each rat on the complete ration received 0.5 cc. of cod liver oil daily, administered by means of a pipette. The average coefficients of digestibility found in those animals on the vitamin D-deficient diet were as follows: 88 for protein, 83 for fat, and 93 for carbohydrate. For those animals receiving vitamin D the coefficients of digestibility were 89 for protein, 96 for fat, and 93 for carbohydrate.

In Experiment II, the rats which received cod liver oil showed much higher coefficients of digestibility of fat than did the controls. Since no such difference was shown in Experiment I, in which no cod liver oil was given, it is evident that the high coefficient was caused by a loss of oil during its administration.

Average coefficients of digestibility as determined in the two experiments are:

	No vitamin D	Vitamin D
Protein....	87	88
Fat....	84	88
Carbohydrate....	93	92

The coefficients of digestibility for protein, fat, and carbohydrate appear to be the same in those animals which received vitamin D as in those which were on the vitamin D-deficient diet.

SUMMARY

A comparison has been made of the metabolism of animals on a diet deficient in a given vitamin with the metabolism of animals upon the same diet supplemented with the missing vitamin. Such a study has been made with each of the vitamins, vitamins A, B_1, B_2, C, and D. In the case of each of these vitamins, the coefficients of digestibility of protein, fat, and carbohydrate were the same in those animals on the deficient ration as in those which

received the complete diet. It would appear unlikely, therefore, that the digestive process is affected by the vitamins studied.

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THE RÔLE OF IRON AND COPPER IN THE GROWTH AND METABOLISM OF YEAST

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Most synthetic media for the growth of yeast have been compounded with little consideration of the presence of such elements as iron and copper. The work of Warburg (1), which has shown that respiration in most living cells is due to a process catalyzed by iron, naturally attracts attention to the iron requirements of living cells. Yeast has been used to such a large extent in the experimental studies on the mechanism of respiration that an investigation of the effect of iron on the metabolism of this organism should prove valuable.

Harpuder (2), while studying the effect of iron and manganese on the respiration and fermentation of yeast, found that $FeSO_4$ in concentrations of 10^{-4} to 10^{-5} molar increased respiration in bakers' yeast, and that concentrations of 10^{-3} decreased fermentation. The respiration of beer yeast was not affected by iron, but the fermentation was decreased when 10^{-3} molar $FeSO_4$ was added. $MnSO_4$ had no effect on the respiration of bakers' or beer yeast, though a concentration of 10^{-3} to 10^{-6} molar $MnSO_4$ increased the fermentation of bakers' yeast. Von Euler and Eriksson (3) found that the cultivation of yeast in media containing 1 gm. of $FePO_4$ or 2 gm. of $FeSO_4$ per liter increased the total iron content of the yeast. The yeast's capacity for reducing methylene blue and consuming oxygen decreased with increased iron content. The conditions in both these investigations differed from those under which yeast is normally grown. In Harpuder's work the yeast was in contact with the metal only a short time, while in von Euler's experiments, although the yeast was cultivated for 5 days in the iron-rich media, exceedingly high amounts of iron were used.

Bortels (4), investigating the importance of iron, zinc, and copper, for the growth of microorganisms (especially *Aspergillus niger*), purified all his solutions very carefully before inoculation and added the metals to be

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tested in small amounts. He found that iron and zinc were necessary for the growth of *Aspergillus niger* and that copper stimulated the formation of the black conidial pigment of the mold. Although his experiments with yeast were few, he concluded that both iron and zinc stimulate the growth of this organism but that copper is without effect.

Warburg's evidence (5) that the respiratory enzyme, or das Atmungsferment, is an iron compound, is based largely on the inhibiting action of KCN on cell respiration. Since Negelein (6), and Dixon and Elliott (7) have shown that cyanide produces a practically complete inhibition of the respiration in yeast, it is clear that the study of the effect of the iron content of the medium on the respiration of yeast becomes as important as its effect on the total growth of the cells.

The more recent work of Warburg (8), concerning the depressing action of carbon monoxide at high partial pressures on the oxygen uptake of living cells, has led him to believe that the respiratory enzyme is a hematin compound. However, Keilin (9) has demonstrated that the respiratory mechanism of yeast does not consist of a single iron complex, but that at least part of the iron is present as cytochrome which acts as an intermediate carrier between the catalytic systems which reduce it and those which oxidize it. The dehydrogenase systems are responsible for the reduction of cytochrome and the indophenol oxidase functions in its oxidation. Cyanide and carbon monoxide therefore inhibit cell respiration because they prevent the activity of the indophenol oxidase in the oxidation of cytochrome. Keilin (10) has shown that cytochrome is not one compound but a mixture of three independent hemochromogen-like pigments, which give characteristic absorption spectra when in the reduced state. It is, therefore, also interesting to study the effect of media on the formation of these hematin compounds in yeast.

Since it has been demonstrated by Hart, Steenbock, Waddell, and Elvehjem (11), and Elvehjem and Hart (12), that copper is necessary for hemoglobin formation in higher animals, a study of the effect of copper on the formation of the hematin compounds in yeast may throw some light on the mode of action of copper in the animal body.

It is my purpose in this paper to present some of the more general facts observed during a study of the growth, respiration, cytochrome content, and iron content of yeast grown on media containing varying amounts of iron and copper. Some of the results need to be investigated in greater detail and will form the basis of further work.

<center>EXPERIMENTAL</center>

The medium used for the growth of yeast consisted of sucrose, 2.5 per cent; $MgSO_4$, 0.25 per cent; KCl, 0.25 per cent; NH_4Cl, 0.25 per cent; Na_2HPO_4, 0.25 per cent; and $CaCl_2$, 0.05 per cent.

This is essentially Wildiers' medium (13) except that the percentage of sucrose is reduced from 10 to 2.5 and the $CaCO_3$ is replaced by $CaCl_2$. Qualitative tests for iron and copper showed that traces of both these metals were present in each of the ingredients; the salts and sugar were therefore purified so that the final medium would be iron- and copper-free. All the water used in these experiments was redistilled from an all glass apparatus. The glass utensils were always cleaned with chromic acid solution, washed with HCl, and rinsed with redistilled water.

The constituents of the medium were purified in three parts. In Part I, 10 gm. each of KCl, NH_4Cl, and Na_2HPO_4, and 100 gm. of sucrose, were dissolved in about 350 cc. of water, and sufficient $CaCl_2$ solution added to produce a noticeable precipitate of calcium phosphate. The pH of this solution was 7.8, and at this alkalinity the iron and copper are precipitated as phosphates and carried down by the calcium phosphate which is formed. The precipitate was allowed to settle overnight, filtered off, and the filtrate made to 400 cc. In Part II, 10 gm. of $MgSO_4$ were dissolved in about 40 cc. of water and sufficient Na_2HPO_4 solution added to produce a definite precipitate of magnesium phosphate. In Part III, 2 gm. of $CaCl_2$ were added to about 40 cc. of water and Na_2HPO_4 solution added to produce a slight precipitate of calcium phosphate. In the case of the last two solutions a small amount of 0.2 N NaOH was added to bring the pH to 7.8. The precipitate in both cases was filtered off after 24 hours and each filtrate made to 50 cc. 2 liters of medium were then made from these purified solutions by diluting 200 cc. of Solution I, 25 cc. of Solution II, and 25 cc. of Solution III, to 2000 cc. Analyses showed that 200 cc. of this medium contained less than 0.005 mg. of Fe and less than 0.002 mg. of Cu.

The different media were always inoculated with yeast, which had been grown for at least one subcultivation on a purified medium, in order to eliminate any effect of stored iron and copper present in the commercial yeast. The cells when 48 hours old were centrifuged off, washed with distilled water, and made to a volume so that 1 cc. contained 10 mg. of dry yeast. A new purified medium was seeded every 48 hours in order to have a continuous supply of yeast available for inoculations. At least 30 separate cultures of yeast were subcultivated under the final

growing conditions with no change in the rate of growth, which shows that the same growth-influencing factors were added with each seeding. The original samples of commercial bakers' yeast used varied in iron content from 0.08 to 0.20 mg. of Fe, and in copper content from 0.04 to 0.06 mg. of Cu per gm. of dry matter.

The first measurements were made with yeast grown in 200 cc. of medium contained in 1 liter Erlenmeyer flasks and incubated at 35°. A stream of air was passed through each flask continually. The final studies were made on yeast grown in 500 cc. flasks containing 200 cc. of the medium, which were aerated at a tem-

TABLE I

Effect of Iron and Copper on Growth of Yeast

Culture No.	Yeast used for inoculation, dry weight	Age	Temperature	Dry weight of yeast in 200 cc. medium		
				Alone	Fe	Fe and Cu
	mg.	*hrs.*	*°C.*	*gm.*	*gm.*	*gm.*
16	15	45	35	0.128	0.320	0.403
18	20	42	35	0.172	0.400	0.186*
19	10	48	35	0.080	0.184	0.236
20	20	48	25	0.222	0.536	0.670
23	20	40	25	0.176	0.544	0.708
30	20	42	25	0.156	0.548	0.564
30	20	90	25		0.840	0.960
30	20	168	25	0.224	1.048	1.200

*Copper alone without iron.

perature of 25°. In most cases 200 cc. of medium were inoculated with a 2 cc. suspension of cells equivalent to 20 mg. of dry yeast. 1 drop of olive oil was added to each flask to prevent frothing.

Growth of Yeast

The rate of growth was determined by the weight of the yeast produced in definite periods of time. The cells in 25 or 50 cc. of medium were centrifuged off, washed twice with distilled water, dried, and weighed. The growth was studied mainly in three types of media; purified media, purified media plus 0.2 mg. of Fe per 200 cc., and purified media plus 0.2 mg. of Fe and 0.02 mg. of Cu per 200 cc.

The figures in Table I represent the dry weights per 200 cc. of

medium of yeast grown with different amounts of metals in media aerated at 35° and at 25° respectively. It is readily seen from these results that yeast makes but a meager growth on a medium free from iron and copper. Regardless of the conditions under which the organism is grown, there is never more than a 10-fold increase in the weight of the cells during the first 48 hours. As soon as a small amount of iron is added there is a decided increase in the rate of growth; the total weight at the end of 48 hours is 2 to 5 times as great with iron as without. This shows that iron is definitely a limiting factor in the purified medium, and that this element is essential for the growth of yeast. The difference in growth is most evident in samples grown at 25° because at 35° the temperature is a limiting factor. Richards (14) has shown that temperatures above 30° retard the growth of yeast. The presence of copper in addition to iron gives a still greater increase in the rate of growth. This further stimulation is also most noticeable at 25° when the average growth is 25 per cent greater in the presence of copper than in its absence. Whether the additional growth would be still greater should other limiting factors be removed remains for further investigation. The results in Table I also show that copper alone gives no growth stimulation, which further demonstrates the importance of iron and shows that the activity of the copper is dependent upon the presence of iron.

Properties of Anemic Yeast

Yeast not only grows very slowly in media devoid of iron and copper, but the cells which are produced are entirely unlike normal bakers' yeast and the low pigment content of these cells may be compared in certain respects to the condition of anemia in animals. The yeast produced on the purified medium is exceedingly white and colorless; even when dried it exhibits only a slight yellowish color, while ordinary yeast has a distinct brown color. The total iron content, as determined by the modified thiocyanate method, Elvehjem (15), was found to vary for the different samples from 0.03 to 0.06 mg. of Fe per gm. of dry yeast. These figures are one-third to one-half of those for normal yeast. Free inorganic iron was measured with α,α-bipyridine[1] according to the method

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[1] I am indebted to Mr. R. Hill for supplying me with a sample of α,α-bipyridine.

of Hill (16), and no trace of inorganic iron could be detected in the yeast grown on the purified media, though definite amounts could be demonstrated in the cells grown on the supplemented media. The last traces of inorganic iron had undoubtedly been utilized for the formation of hematin compounds necessary for the life processes of the cells.

The cytochrome content determined microspectroscopically was found to be exceedingly low; much less than the amount present in the yeast to which iron additions had been made. It is interesting to note, however, that a feeble cytochrome spectrum could be detected which shows that although the amount of this pigment may be reduced, a certain amount must be present before growth can take place. The respiration of this yeast was also found to be very low; samples 48 hours old gave a Q_{O_2}[2] value of 9 at 20°. When 96 hours old the value had increased to 18, and one sample 144 hours old reached the value of 31. The gradual increase in the respiratory rate with age demonstrates that the cells slowly convert every available supply of iron into the more active compounds necessary for energy production.

Respiration of Yeast

The total respiration as well as the cyanide-stable respiration of the yeast grown under different conditions was measured, when the cultures had been incubated for varying periods of time, in differential Barcroft micro respirometers. Each manometer was properly calibrated before use. A volume of medium known to contain a definite weight of yeast was centrifuged to remove the cells from the original liquid. The cells were washed twice with distilled water and suspended in a definite volume of 0.05 M Na_2HPO_4 buffer, adjusted to pH 7.0, containing $\frac{1}{18}$ M glucose. After $\frac{1}{2}$ hour, 3 cc. of this suspension were placed in the right-hand flask, and 3 cc. of phosphate buffer alone were added to the left-hand flask, of each manometer. When KCN was used, 0.3 cc. of the solution in each flask was replaced by 0.3 cc. of 0.1 M KCN which was neutralized to pH 7.0 directly before it was used. The small tubes within each flask contained rolls of filter paper mois-

[2] Q_{O_2} in all cases indicates the c.mm. of O_2 taken up per mg. of dry yeast per hour as measured in air.

tened with 6 per cent KOH to absorb any CO_2 produced during respiration.

The manometers were shaken in a water bath at 20° and after 5 minutes equilibration the taps were closed and readings taken at definite intervals. The Q_{O_2} for each sample was calculated from the oxygen uptake and the dry weight of the yeast used. By using this procedure the respiration of all samples was measured under standard conditions, and thereby errors due to changes in the supply of unoxidized sugar, or to the presence of additional metabolites produced during the decomposition of the sugar, were eliminated.

The respiration of samples of yeast grown on different media at 35° and 25° are given in Table II. It has already been men-

TABLE II

Respiration of Yeast

Culture No.	Age	Temperature	Q_{O_2} values for yeast from different media		
			Purified	Fe	Fe and Cu
	hrs.	°*C.*			
17	42	35	11	16	21
17	70	35	15	26	32
18	42	35	8	18	19
23	40	25	9	39	37
30	42	25	11	39	39
30	90	25	19	33.	31
30	168	25	31	14	17

tioned that the Q_{O_2} values for yeast from purified media are low at 42 hours (8 to 11) but slowly increase as the yeast becomes older. Samples from the iron, and the iron plus copper, cultures grown at 35° also show rather low values at 42 hours (15 to 20) but give somewhat higher values at 70 hours (25 to 30). The low figures at 42 hours are undoubtedly due to a lack of available iron since the pH of the original medium is about 7.0 and the iron remains insoluble until acidity is developed. As growth takes place the culture becomes more acid making the iron available for the formation of hematin compounds. Hopkins and Wann (17) obtained similar results when studying the availability of iron for the growth of *Chlorella*. The respiration is slightly higher for the yeast grown in the presence of copper because growth was more

rapid in the presence of this element and acidity developed sooner.

The figures for the oxygen uptake of the yeast produced on the supplemented media at 25° are considerably higher. Q_{O_2} values of 40 were often obtained at the end of 48 hours because growth was more rapid and the iron became available for respiratory pigment formation much earlier. This value is considerably higher than that obtained by Warburg (18) for yeast of the same age grown on beer wort. His figures correspond more closely to those for the yeast grown at the higher temperature or the yeast produced in the absence of iron. We shall see later that his results can probably be explained by a low iron content of the beer wort used.

The majority of all the Q_{O_2} values for yeast grown on the supplemented media at the lower temperature fall between 35 to 40 at 48 hours of age; in some cases the values for the yeast receiving both iron and copper are slightly lower. This difference may be explained by changes in the type of cytochrome, and will be discussed later. Although these figures are very high for young cells, they do not reach the values reported for commercial bakers' yeast. The value for commercial yeast determined by the method used in this work was found to be 85. Warburg found the respiration to increase during aeration of the yeast; therefore, attempts were made to obtain similar results when the yeast was grown on synthetic media.

The yeast was grown for varying periods of time without altering the media in any way, and the respiration studied at definite intervals. Instead of finding a gradual increase in the respiratory rate with continued aeration, a decided decrease in the rate was noted. In practically every case the yeast grown on the supplemented media gave Q_{O_2} values of 15 to 20 after 6 to 7 days while the yeast on the purified media gave increasing values during the same time and reached values of 30. When the different cultures were examined for acidity it was found that the pH of the supplemented media decreased to 2 within 60 hours while the pH of the purified media never fell below 3.6 during this time. The relation between decrease in respiration and acidity was therefore investigated. The following work will show that high acidity is the main factor in the destruction of the major respiratory mechanism, but that cytochrome remains unaffected during this change.

Cytochrome Content of Yeast

All samples of yeast grown on the different media for varying lengths of time were examined microspectroscopically for cytochrome. The cytochrome content of the yeast grown on the purified media was low at 42 hours, but increased slightly with age, the increase in every case being accompanied by a larger respiratory rate. This demonstrates that cytochrome is the limiting factor in the respiration of this yeast. The increase in cytochrome content could be detected when the yeast was stored in an ice chest for several days as well as when it remained in the culture media.

The yeast produced on the purified media plus iron gave a cytochrome spectrum quite similar to that of commercial yeast at the end of 48 hours and the amount increased very little per unit of cell material as aeration was continued. This proves without question that yeast cells can synthesize cytochrome directly from inorganic iron. The cytochrome in the yeast incubated for 7 days in the original media, which had become very acid, exhibited no changes that could be detected spectroscopically. This indicates that the decrease in respiration is not due to any change in this part of the respiratory mechanism.

The cytochrome spectrum of the yeast grown with both iron and copper was decidedly different in character. The *b* and *c* bands remained the same or decreased slightly in magnitude but the *a* band became much more definite.[3] Every sample grown in the presence of copper could be detected at once by the increased amount of the component *a* of cytochrome. A difference in the color of the yeast itself was also evident. A mass of cells from the iron media was pinkish in color, while the cells from the iron and copper media exhibited a dark yellowish color. Copper in minute amounts has the property of stimulating cytochrome *a* formation.

This observation is most interesting, when considered in relation to the action of copper in hemoglobin synthesis in the animal body, because the cytochrome of yeast and the hemoglobin of the body undoubtedly originate from the same precursor, protohematin. In the yeast traces of copper bring about changes in the iron compounds which lead to the development of larger amounts of the

[3] See Keilin, D., *Proc. Roy. Soc. London, Series B,* **98,** 312 (1925).

component a of cytochrome and in the animal body minute amounts of copper produce changes necessary for the formation of the final hemoglobin molecule.   The action of copper in the two chemical changes may be very similar.

Copper alone seems to possess the property of stimulating the formation of the component a because the addition of other elements such as zinc and manganese in considerably larger quantities produced no change in the type of cytochrome.   The specificity of copper in this reaction is in accord with the specificity demonstrated for this element for hemoglobin formation by Waddell, Steenbock, and Hart (19).
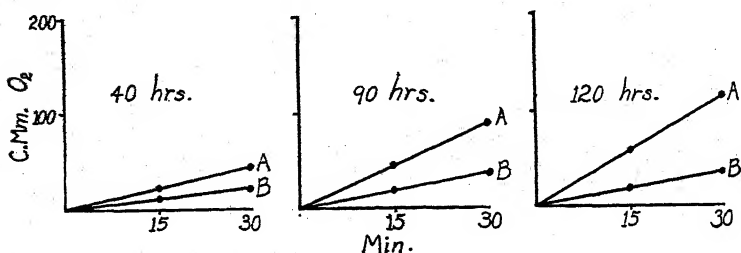
FIG. 1. Oxygen uptake of yeast (10 mg. of dry weight) grown on purified media alone.   Curve A without KCN; Curve B with 0.01 M KCN.

Cyanide-Stable Respiration

That cyanide produces a practically complete inhibition of the respiration in bakers' yeast is an established fact.   0.01 M KCN produced an inhibition of 97.5 per cent in the respiration of the samples of commercial yeast used in this work.   However, the respiration of many of the samples of yeast grown on the synthetic media was inhibited to a much smaller degree.   The percentage inhibition was about 80 in most samples at 48 hours, but the amount of inhibition decreased with age and often the respiration in samples 5 to 7 days old was entirely unaffected by the presence of cyanide.   Figs. 1 to 3 show the total respiration and the cyanide-stable respiration of yeast grown on the different media for varying lengths of time.

In the case of the yeast grown on the purified media the cyanide-stable fraction constituted a large part of the total during the first 3 to 4 days because the entire respiration was low.   As the total

respiration increased the percentage inhibition increased and at least 80 per cent was inactivated by cyanide after 5 days. The total respiration of the yeast grown on the supplemented media decreased, and the cyanide-stable fraction increased with age so that at 120 hours at least 75 per cent was unaffected by KCN. After 6 days the entire oxygen uptake was not sensitive to 0.01 M KCN.
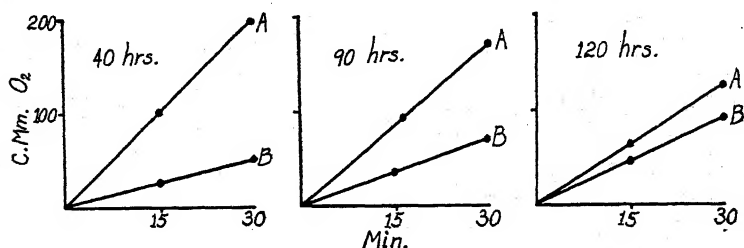
FIG. 2. Oxygen uptake of yeast (10 mg. of dry weight) grown on purified media plus iron. Curve A without KCN; Curve B with 0.01 M KCN.
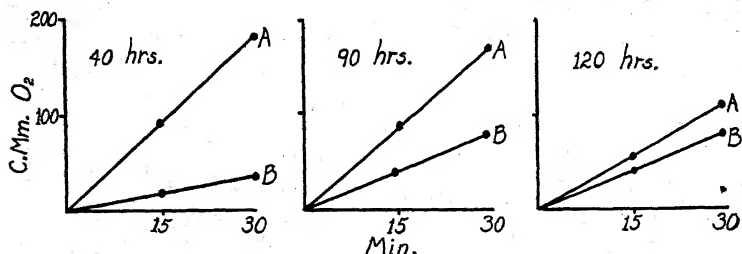
FIG. 3. Oxygen uptake of yeast (10 mg. of dry weight) grown on purified media plus iron and copper. Curve A without KCN; Curve B with 0.01 M KCN. The respiration in each case was measured in glucose phosphate buffer at 20°.

That part of the respiratory mechanism which is inhibited by cyanide seems to be destroyed or altered by the acidity, and the yeast must depend upon another system or a modified system for the production of energy. The new system is not as efficient because the rate of respiration is decreased considerably. Q_{O_2} values for yeast stable to cyanide never exceeded 16 to 17. The high acidity must be the main factor in bringing about this change because the KCN-labile respiration is not destroyed in the yeast

produced on a purified medium, which never reaches pH values below 3.6.   The change cannot be due to a preponderance of a different strain of yeast in the more acid media because when new media were inoculated with this yeast the respiration of the new yeast was again KCN-sensitive until the new media developed high acidity.

Some of the properties of the cyanide-stable system may be gained from Fig. 4.   These measurements were made on Culture 22, consisting of the purified media plus iron and copper, when the yeast was 120 hours old and the respiration was practically unaffected by 0.01 M KCN.   Respiration was reduced to 10 per cent of the total when the cells were heated to 60° for 5 minutes, which
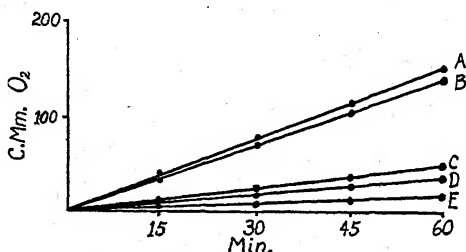
FIG. 4. Properties of cyanide-stable respiration.   Oxygen uptake of yeast (10 mg. of dry weight) grown on purified media plus iron when 120 hours old.   Curve A alone; Curve B with 0.01 M KCN; Curve C plus 5 per cent urethane; Curve D in phosphate buffer without glucose; Curve E with cells heated to 60° for 5 minutes.

shows that the system is thermolabile.   The system metabolizes glucose since the oxygen uptake was reduced to 25 per cent when the determination was made in the phosphate buffer alone.   Although the system is not inhibited by KCN it is inhibited to an extent of about 70 per cent by 5 per cent urethane.   These facts suggest that the acidity has destroyed the indophenol oxidase present in yeast, and that the dehydrogenase systems and the cytochrome are still present.

Effect of Reaction of Media on the Cyanide-Labile Respiration

The results presented thus far demonstrate that the pH of the media has an important effect on the respiration of the yeast and that widely divergent rates of respiration may be recorded de-

pending upon the conditions operative in the culture media. Since the synthetic media used developed acidity with the continued growth of the yeast, attempts were made to use cultures which would eliminate this difficulty. Wildiers' medium was used directly without purification of the constituents so that $CaCO_3$ could be incorporated in the salt mixture, but it was found that even the amount of $CaCO_3$ called for in this medium was insufficient to prevent the rapid development of acidity when yeast equivalent to 20 mg. of dry yeast was used for inoculation. The use of beer wort was found to be the most satisfactory means of regulating the H ion concentration. The pH of the original wort used was 5.2, but decreased to 3.8 during the first 48 hours of yeast growth

TABLE III

Comparison of Yeast Grown on Wildiers' Medium and on Beer Wort

The initial inoculum was 20 mg. of dry yeast per 200 cc. of medium.

Medium	Age	pH	Dry yeast per 200 cc.	Respiration Q_{O_2}		Per cent not inhibited by KCN
				Without KCN	With KCN	
	hrs.		*gm.*			
Wildiers'......................	40	2.8	0.744	24	2.0	8
"	64	2.2	0.888	31	3.5	12
"	136	1.8	1.232	22	13.0	59
Beer Wort I...................	40	3.8	0.912	15	0.6	4
"	64	3.8	1.008	19	1.0	5
"	136	3.8	1.168	25	3.5	14

and remained at that figure during the rest of the incubation. Table III gives a summary of the results obtained when Wildiers' medium and beer wort were used for the growth of yeast.

The figures for Wildiers' media are practically identical to those obtained for yeast produced on the supplemented purified media. The pH reaches a low level very early and the Q_{O_2} value is 31 at 64 hours with only 12 per cent not inhibited by KCN, but decreases to 22 at 136 hours and 59 per cent is cyanide-stable. The results for yeast grown with beer wort are quite different. The pH remains at 3.8, the Q_{O_2} values are very low at first but gradually increase as the cells become older, and more than 80 per cent of the respiration is inhibited by KCN even after 6 days. In other

words, when the development of acidity is retarded practically all the respiration is cyanide-labile. The total respiration for the beer wort yeast, however, is low and corresponds to that found by Warburg. The explanation for the low results became evident when the wort was analyzed for iron and found to be exceedingly low, only 0.079 mg. of Fe in 200 cc. of medium. Every trace of iron would have to be utilized in order to give a normal amount of iron in the yeast produced. The cytochrome content of this yeast was also low, showing that there was insufficient iron in the beer wort for the production of proper amounts of hematin compounds.

A study of the distribution of iron in a sample of beer wort during the growth of yeast is shown in Table IV. The wort was analyzed before inoculation and the yeast and remaining wort were

TABLE IV

Distribution of Iron in Medium and Yeast

	mg.
Fe in 200 cc. beer wort....................................	0.112
" added in 20 mg. yeast for inoculation....................	0.001
Total...	0.113
Fe in medium after growth of yeast........................	0.056
" removed by yeast......................................	0.057
" content of yeast by analysis..........................	0.065

analyzed when the culture was 120 hours old. This sample of wort contained more iron than the first sample, an amount sufficiently greater to produce more cytochrome and a correspondingly higher respiratory quotient, but the results show that even this sample was low in iron and that a large part of the total had to be assimilated in order to give the yeast a proper supply of iron.

Effect of Bios on Respiration

Copping (20) has shown that the addition of bios to cultures of *Saccharomyces cerevisiæ* greatly stimulates growth and seems to provide the organism with the power to respire oxygen. She suggests that it is impossible from her experiments to determine whether the increased respiratory activity of the yeast is due to the greater number of cells present or to some change in their

metabolic processes.   The effect of bios on the growth and the respiration of yeast grown on the cultures used in this work was therefore studied.

The source of bios was a yeast extract which was kindly furnished by Dr. Guha, who prepared it in the following way: 3.2 kilos of top brewers' yeast were extracted with 1250 cc. of 97 per cent alcohol at 50–60° for 3 to 4 hours.   The residue was reëxtracted with 1600 cc. of 50 per cent alcohol under the same conditions.   The combined filtrates were treated with 500 cc. of saturated lead acetate, the precipitate filtered off, and the lead removed from the filtrate with H_2S.   The solution was concentrated so that 1 cc. was equivalent to 4.6 gm. of fresh yeast.   1 cc. of this solution was added to 200 cc. of culture medium.

TABLE V

Effect of Bios on Growth and Respiration of Yeast

Medium	Age	Dry yeast per 200 cc.	Respiration Q_{O_2}		Per cent not inhibited by KCN
			Without KCN	With KCN	
	hrs.	*gm.*			
Wildiers'.............................	40	0.848	36	6.0	17
"	64	1.264	38	11.8	31
"	112	1.480	35	24.0	69
" + bios...................	40	1.048	31	10.0	32
" + "	64	1.336	37	16.3	44
" + "	112	1.320	35	30.5	87

Results in Table V show that the addition of bios to the unpurified Wildiers' medium stimulated the rate of growth but did not affect the final amount of yeast produced.   The magnitude of the stimulation was smaller than that generally recorded when bios is added to synthetic media, most probably due to the rather large amounts of yeast used for inoculation.   The cultures used in this work, therefore, have not been decidedly deficient in the bios factor.   No difference was detected in the total respiratory rates for the yeast grown in the presence of additional bios and that grown in its absence.   The cyanide-labile respiration decreased at a faster rate with age in the yeast grown with bios than without; 87 per cent was cyanide-stable in the former and only 69 per cent

in the latter at 112 hours.   The more rapid destruction of the
cyanide-labile fraction in the case of the bios culture is due to the
more rapid growth and faster development of acidity.   These
results emphasize the necessity of controlling the acidity of the
media if the effect of such factors as bios on the respiratory activity
of yeast is to be studied.

Similar results were obtained when bios was added to the
purified media supplemented with iron and copper; a large portion
of the respiration was cyanide-stable after 4 days of growth.   If
on the other hand, these media were carefully neutralized with
sodium hydroxide at short intervals so that the pH never fell
below 4.0, 80 per cent of the respiration was inhibited by KCN
even after 6 days and the total respiration reached higher values.
The results are given in Table VI and show that Q_{O_2} values com-

TABLE VI

Effect of Neutralization of Medium on Respiration of Yeast

Medium	Age	Dry yeast per 200 cc.	Respiration Q_{O_2}		Per cent not inhibited by KCN
			Without KCN	With KCN	
	hrs.	*gm.*			
Purified + Fe + Cu + bios	92	0.320	91	10	11
neutralized to pH 4.0 at	144	0.624	53	11	21
short intervals					

parable to those for commercial yeast were obtained.   The growth
was retarded to some extent, very probably due to the continual
addition of NaOH.   However, the results demonstrate that
synthetic media will produce yeast with respiratory values equi-
valent to normal bakers' yeast providing the proper conditions
prevail.   The exact methods of controlling these conditions, and
the part that bios plays in the formation of the respiratory mech-
anism, must be studied in greater detail.

Effect of Larger Additions of Copper

All the media containing copper, which have been discussed
thus far, have contained 0.02 mg. of Cu per 200 cc. of medium.
Since this amount definitely stimulated the formation of cyto-
chrome *a*, it seemed advisable to determine whether the addition

of larger amounts of copper would produce any further change. Yeast grown on media containing 4 and 16 times as much copper, respectively, was compared with that produced from media containing the smaller amount of copper. The results are given in Table VII. The addition of 0.32 mg. of Cu had a distinct inhibitory action on the development of the yeast, the growth at 40 hours was less than one-third the amount produced with the smaller amount of copper. The inhibition with 0.08 mg. of Cu was only slight. At 108 hours the growth was no longer inhibited and the amount of yeast in all the cultures was about the same. The toxic effect of the copper disappears as the number of yeast cells increase. This result is in accord with the work of Voegtlin, Johnson, and Dyer (21), and Flinn and Inouye (22) on the toxicity

TABLE VII

Effect of Increased Amounts of Copper

Medium	Age	Dry yeast per 200 cc.	Respiration Q_{O_2}
	hrs.	*gm.*	
Purified + Fe +0.02 mg. Cu	42	0.704	26
" + " +0.02 " "	108	1.130	18
" + " +0.08 " "	42	0.600	22
" + " +0.08 " "	108	0.940	18
" + " +0.32 " "	42	0.224	22
" + " +0.32 " "	108	0.804	24

of copper. The respiration of the yeast grown with varying amounts of copper exhibited no striking differences. The cytochrome *a* content was no higher in the presence of the larger quantities of copper than with 0.02 mg. of Cu, which shows that the smaller quantity is the optimum amount for stimulating the formation of this component of cytochrome.

Iron Content of Yeast

The total iron content was determined after ashing by the modified thiocyanate method, Elvehjem (15), and tests for free inorganic iron were made by the method of Hill (16). Figures for the iron content of yeast grown on the three different media when 40 hours and 156 hours old are in Table VIII. The yeast grown on the purified media has a low iron content regardless of the

length of cultivation.   The iron content of the yeast grown on the purified media supplemented with iron or both iron and copper is quite high, somewhat higher than most samples of commercial yeast.   The higher value is due to the rather liberal supply of iron (0.2 mg. of Fe per 200 cc. of medium).   When the cultures get older the iron content decreases because the iron available per unit of cell material becomes less as the amount of yeast increases.

It has already been mentioned that no inorganic iron can be detected in the cells grown on the purified media, but definite quantities can be demonstrated in the cells produced on the supplemented media.   No attempt was made to obtain exact quantitative figures for the inorganic iron in the yeast showing the higher iron content, but at least one-half of the total was present in the inorganic form.   The inorganic fraction decreased as the total iron decreased.

TABLE VIII

Total Iron Content of Yeast Grown on Different Media

Medium	40 hrs. old		156 hrs. old	
	Dry yeast per 200 cc.	Fe per 1 gm. yeast	Dry yeast per 200 cc.	Fe per 1 gm. yeast
	gm.	*mg.*	*gm.*	*mg.*
Purified...........................	0.176	0.05	0.222	0.06
" + Fe..........................	0.544	0.23	1.072	0.14
" + " + Cu....................	0.708	0.24	1.432	0.12

Studies on the respiratory rates of the yeast have indicated that respiration is limited during the early period of growth, when the pH of the medium is still rather high, by a lack of available iron. The rate at which iron was assimilated from buffer solutions of different H ion concentrations by anemic yeast was therefore investigated.   Anemic yeast equivalent to 50 mg. of dry yeast was suspended in 25 cc. of buffer solution containing Na_2HPO_4 and $CaCl_2$, in the same concentration as the synthetic media, and 0.05 mg. of Fe at pH 7.0 and at pH 4.0.   After the cells had been in contact with the iron for 8 hours, they were centrifuged off, washed very thoroughly, and suspended in sodium acetate buffer.   Upon the addition of bipyridine and sodium hydrosulfite, the cells kept at pH 4.0 assumed a pinkish color, but those kept at pH 7.0 remained

completely colorless. The cells at the lower pH had assimilated the iron but those at the higher pH were unable to obtain any from the buffer solution. This result shows that pH 7.0 is obviously not the optimum H ion concentration for the growth of yeast because under these conditions the iron is insoluble and unavailable. A lower pH, at which the iron is more soluble, will undoubtedly allow greater growth of yeast providing the pH is not low enough to destroy certain constituents of the respiratory mechanism. Darby (23) has found that the optimum pH for the growth of yeast is 4.4.

<div align="center">DISCUSSION</div>

The fact that the addition of a small quantity of yeast extract to synthetic media made up of purified salts and sugar together with traces of iron and copper produces a stimulation in the rate of yeast growth shows that all the conditions for optimum growth are not present. A discussion of all the views regarding bios and its importance as an essential for the growth of yeast cannot be made here, but it is interesting to consider whether any of the results presented in this paper can be used to explain any of the many contradictory conclusions previously made in regard to bios. Although the work presented here has shown that iron is essential for the growth of yeast and that media can be prepared so low in this element that growth is retarded, it is doubtful if any of the synthetic media used by the different investigators have been sufficiently lacking in iron to prevent normal growth. Most of the salts used for the preparation of synthetic media contain large enough quantities of iron as an impurity to supply the necessary amount.

The total supply of iron may not be of importance but the availability of this element is of greater significance and is a limiting factor in the determination of the rate of yeast growth especially if the pH of the medium remains rather high. A considerable part of the beneficial action of bios on yeast growth is probably dependent upon changes which make the iron more available for assimilation under conditions of high pH. In fact, Darby (23) suggests that the observation of Wildiers is entirely explicable by the buffering action of the organism on a hyperalkaline medium, that the original pH of Wildiers' medium was

approximately 7.2; the optimum for growth is 4.4.   Bios produced very little growth stimulation in the media used in the work reported here because the increased sugar metabolism due to the rather large inoculum used reduced the alkalinity to the optimum pH very early in the growing period.   When the medium is maintained at the proper pH the activity of the bios is not so noticeable. More work is required before it will be possible to determine whether the effect of bios can be eliminated entirely by regulation of acidity or the bios actually stimulates yeast growth, but in either case the effect will be closely associated with the availability of the iron.

The copper content of ordinary synthetic media is very probably never a limiting factor in the growth of yeast because even the addition of such traces as 0.02 mg. of Cu per 200 cc. of medium, although stimulating growth, produces yeast containing an abnormal amount of cytochrome *a*.   When our methods are improved still further we may find that the purified media used in this work contained sufficient copper for normal metabolism, and that the addition of smaller amounts of copper to a more purified medium will show beneficial effects without changing the type of cytochrome.

SUMMARY

1. A method for the preparation of a synthetic culture medium which is exceedingly low in iron and copper is described.

2. Bakers' yeast makes a meager growth on a medium low in iron and copper and the yeast has the following characteristic properties: pale color, low total iron content, complete absence of free inorganic iron, low cytochrome content, and low respiratory quotient.

3. The addition of a small amount of iron accelerates the rate of growth, increases the cytochrome content to the amount normally present in commercial yeast, and increases the respiratory quotient to Q_{O_2} values of 35 to 40.   Iron is therefore an essential element for the growth and metabolism of yeast and can be used directly for cytochrome synthesis.

4. The addition of both iron and copper gives a further increase in the rate of growth and causes the production of cytochrome with a distinctly higher *a* component.   Copper has the property of stimulating the formation of certain hematin compounds.

5. Q_{O_2} values of 35 to 40 observed when the yeast is 48 hours old decrease with continued aeration of the medium to 15 to 20 when the culture is 6 to 7 days old due to the high acidity of the medium. At least 80 per cent of the respiration is inhibited by KCN at 48 hours of age but the KCN-labile portion gradually decreases and after 7 days the oxygen uptake is completely unaffected by KCN. When the reaction of the medium is properly controlled respiratory rates comparable to those of commercial yeast are obtained.

6. The KCN-stable system metabolizes glucose, is destroyed by heating to 60°, and is inhibited to an extent of 70 per cent by urethane.

7. The respiration of yeast grown on beer wort, which prevents the development of high acidity, is largely inhibited by cyanide. The total respiration of yeast grown on beer wort may be low due to an insufficient supply of iron in the wort.

8. The addition of bios to the synthetic media used in this work stimulated the rate of growth to a small extent, but had no effect on the respiratory activity of the yeast.

9. 0.02 mg. of Cu per 200 cc. of medium is the optimum amount for stimulating the formation of cytochrome a. Additional amounts of copper inhibit cell growth during the first 48 hours, but the toxic effect disappears as the number of cells per unit of medium increases.

10. The iron content of yeast grown on the supplemented media depends upon the ratio of available iron to the amount of yeast present. Yeast low in inorganic iron is unable to assimilate iron from a phosphate buffer at pH 7.0 but the cells readily take up iron at pH 4.0. The availability of iron may be the limiting factor in many of the synthetic media which have been used for studying the growth requirements of yeast.

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THE NATURE OF THE HIGHLY UNSATURATED FATTY ACIDS STORED IN THE LARD FROM PIGS FED ON MENHADEN OIL

By J. B. BROWN

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In preceding reports (1, 2) it was shown that when fish oil was fed to white rats equilibrium between the diet and the depot fat resulted in from 4 to 6 weeks, and further that with a series of diets containing from 5 to 30 per cent of menhaden oil the amount of highly unsaturated acids in the depot fat was roughly proportional to their content in the diet. Relatively more of these acids, however, was found in the fat from animals on the diets of lower oil content. Analysis of the polybromides of the fatty acids from the body fats resulted in each case in higher bromine content than the bromides from the original fish oil acids, suggesting that the deposited acids were more unsaturated than those from the original oil.

In the present investigation the purpose in mind was to feed a larger species of animals on a fish oil diet with the hope of being able to isolate enough of the deposited unsaturated acids to study their properties and ascertain whether they had undergone change during assimilation. Pigs were chosen as experimental animals because they will readily eat food containing large amounts of the oil, and, further, the yield of lard is sufficient for the preparation of the highly unsaturated acids.

EXPERIMENTAL

Experimental Feeding and Preparation of Specimens

The writer is indebted to Professors Joel S. Coffee and F. H. Helmrich of the Department of Animal Husbandry for their interest and cooperation in supervising feeding and slaughtering.

The animals were four Duroc-Jersey pigs 7 months old, two gilts and two barrows. Three of these were litter mates. The control ration was made up as follows: 500 pounds of corn, 45 pounds of tankage, 25 pounds of alfalfa meal, and 8 pounds of salt. The animals were fed for 5 weeks, December 7, 1929, to January 13, 1930. During this period the control pigs ate 330 pounds of the control mixture while the others consumed 270 pounds and in addition 44 pounds of menhaden oil. This oil, a refined specimen purchased on the market, was not unpleasant in taste, aside from its decidedly fishy flavor and odor. Analytically it gave an iodine number of 177.6 and a saponification number of 187.5. Calculated on the basis of 1620 calories per pound of control ration the caloric intake was 16 per cent greater with the oil-fed pigs. Those on the control diet gained 61 pounds during the experiment and those on fish oil 63 pounds. The substitution of oil for part of the control ration was without apparent effect on the health of the animals.

The pigs were slaughtered and cut up under semi-commercial conditions in the abattoir of the Department of Animal Husbandry under the direction of Professor Helmrich. Specimens of back and leaf-fat and the livers and brains were removed from each pig. The lard from the control pigs was rendered in one kettle, while the two specimens from the fish oil pigs were rendered separately in the laboratory.

For convenience in designating the samples, uniform numbering in the data presented below will be used.

Sample F. O. 1 = fed menhaden oil, gilt
    "   F. O. 2 = "   "   " barrow
    "   C. 3   = " control diet, gilt
    "   C. 4   = "   "   " barrow

Results

The following qualitative observations may be of interest. First, during killing the fish oil pigs were decidedly more nervous than the controls. To ascertain whether the fish oil acids had accumulated in the brains, these were analyzed by hydrolyzing in strong alkali, liberating the acids, dissolving in ether, and brominating. The yields of polybromides, however, in both groups of animals were essentially the same. A second difference was observed in the decidedly yellowish color of the carcasses of the fish

oil pigs. Further, the fishy flavor on cooking had penetrated to all parts of the carcass, both the fat and lean portions. It seemed, in fact, to be more concentrated in some of the lean cuts. Even the innermost parts of the tongues were highly flavored. In spite of this, however, the fishy odor in the fresh carcass was scarcely noticeable.

Lipids and Fatty Acids of Livers

The livers were hashed separately, extracted once with 1 volume of boiling alcohol, pressed, and treated again with 2 volumes of the same solvent. The residual proteins were placed in ether for 2 days. The combined alcoholic extracts were concentrated to small volume, the ether extract added, and separated from water. The ether was distilled until foaming became troublesome when 100 cc. of n-butyl alcohol were added. The alcohol (and

TABLE I

Iodine Numbers of Lipids and Fatty Acids of Livers

Sample No.	Lipids	Fatty acids
F. O. 1	113.0	144.5
F. O. 2	129.9	156.4
C. 3	77.2	105.7
C. 4	66.8	119.8

remaining water) was removed by vacuum distillation. The residual lipids were dark amber of soft waxy appearance. The lipids remaining after an iodine number determination were saponified, acidified, and the fatty acids recovered. The results of analysis are given in Table I.

The lipids from the oil-fed pigs gave iodine numbers 49 points higher than the control, while for the fatty acids the oil feeding resulted in an increase of 38 points. For practical reasons the pigs had been kept without food for 24 hours before killing. This increased unsaturation in the liver had persisted therefore for that length of time and might have been considerably greater had the animals been killed immediately. Leathes and Meyer-Wedell (3) described a decided mobilization of unsaturated acids in the livers of cats and rats when fed cod liver oil. They found

that iodine values of liver fatty acids as high as 215 were obtained from oil which had an iodine number of 150 and suggested that the fish oil acids were desaturated by the liver. In view of the fact that the highly unsaturated acids of menhaden (and cod) oil have iodine numbers as high as 388, the increase of unsaturation in the liver may be due merely to selective storage of some of the

TABLE II

Analysis of Lards

Sample	Sample No.	Iodine No.*	Saponification No.
Composite	F. O. 1	74.10	194.7
	F. O. 2	74.68	194.5
	C. 3 and C. 4	65.90	196.1
Back fat	F. O. 1	79.27	194.0
	F. O. 2	78.31	195.0
	C. 3	71.02	196.0
	C. 4	67.60	194.8
Leaf-fat	F. O. 1	67.43	195.2
	F. O. 2	69.05	195.4
	C. 3	62.25	196.3
	C. 4	55.44	195.8

* Hanus, $\frac{1}{2}$ hour reaction.

TABLE III

Analysis of the Methyl Esters of Lard Samples F. O. 1 and F. O. 2

Sample No.	F. O. 1	F. O. 2
Saponification No.	194.7	195.1
Iodine No.	75.05	74.93
Polybromide No.	2.40	2.78
Per cent Br in bromides	69.24	68.67

naturally occurring acids of the fish oil. With this explanation no desaturation is necessary. This question has been recently reviewed by Bloor and Snider (4).

Effect of Menhaden Oil Feeding on the Composition of Lards

The analytical data are given in Table II. The effect of menhaden oil in increasing the unsaturation of the lard and in slightly

lowering the saponification number is apparent, but is much less than was obtained, for example, when white rats were fed (2). On a 15 per cent menhaden oil diet with rats about 15 per cent of highly unsaturated acids were stored, while there was an increase of 26 in the iodine number of the depot fat. The pigs in this experiment consumed a ration containing about 14 per cent of the oil, with an increase of less than 10 in iodine number, while the methyl esters gave polybromide numbers of only 2.40 to 2.78 (see Table III). This diminished effect is due no doubt to the fact that the pigs were not fasted before the experimental feeding began and also to the fact that the metabolic processes of pigs tend

TABLE IV

Analysis of Methyl Esters of Highly Unsaturated Fatty Acids of Lard and of Menhaden Oil

Sample	Menhaden oil	F. O. 1	F. O. 2
Iodine No. of esters	372.1	346.9	329.2
" " " acids*	388.5	361.8	343.7
M. M. Wt. of acids†	302.7	311.6	302.9
Per cent of free acid	2.4	2.1	1.3
Polybromide No.	118.6	89.3	83.9
Per cent Br in bromides	69.72	68.03	68.01

* Calculated.

† Mean molecular weight of the fatty acids, calculated by subtracting $14(CH_2)$ from the molecular weight of the esters.

inherently to synthesize fat which would reduce the total effect of any fatty acid radicals deposited from the diet.

Preparation and Analysis of Highly Unsaturated Fatty Acids Deposited in Lard

The highly unsaturated fatty acids in the lard of the oil-fed pigs were prepared by the usual method of reduction of the methyl ester polybromides in neutral methyl alcohol. The lard was converted into methyl esters by refluxing overnight with $1\frac{1}{2}$ times its weight of anhydrous methyl alcohol, containing 1 to 2 per cent HCl gas. The esters were distilled and brominated in an equal weight of cold ether. After standing several hours, the precipitate was recovered and thoroughly washed with cold ether in 250

cc. bottles, the bromides being separated each time by centrifuga-
tion. After analysis for bromine they were treated with an equal
weight of zinc dust for 18 hours in boiling methyl alcohol. The
alcohol was separated and the residue washed once with hot alcohol
and HCl to decompose any zinc soaps formed during the reaction.
The combined alcohol solutions were treated with additional
HCl gas and refluxed for 5 hours to assure complete esterification.
The esters were recovered and distilled at reduced pressure.
60 gm. of bromides from lard Sample F.O. 1 gave 9.3 gm. of ester,
boiling at 180–220° at 2 to 3 mm.; from Sample F.O. 2, 77 gm. of
bromide gave 9.5 gm. boiling at 200–228° at 7 mm.; 100 gm. of
bromide from menhaden oil gave 15.4 gm. boiling at 180–230° at
3 to 4 mm. The yields were about 50 per cent. The results are
given in Tables III and IV.

In discussing the data in Table IV, it should be recalled that
normal lard contains about 0.4 per cent of arachidonic acid (5),
calculated from the polybromide number of its methyl esters.
The presence of this acid which has about the same molecular
weight and much lower iodine number than the mixed highly
unsaturated acid from menhaden oil will slightly modify the
results.

The highly unsaturated acids stored in the lard of menhaden
oil-fed pigs were much less unsaturated than those from the original
oil, the iodine numbers being 27 to 45 less, in the two preparations.
The molecular weight in one case was the same; in the other it was
9 points higher. Further, both preparations of methyl esters gave
much lower polybromide numbers, the bromides containing less
bromine. These results agree with the general experience of the
writer that with preparations of this kind the polybromide num-
bers increase with the iodine number, one factor in causing this
being the decrease in solubility of the bromides in ether with in-
creased molecular weight or number of bromine atoms in the mole-
cule. The methyl ester polybromides behaved in the melting
point tube like those usually obtained from fish oils. Above 200°
they gradually darkened, and shrank to a black mass with no sign
of melting at 250°. If the deposited acids were composed mainly
of arachidonic acid, the bromides would have melted at 228–230°.

The arachidonic acid present in normal lard could not have
accounted for the differences noted above.

The deposition of 2.7 per cent of highly unsaturated acids in lard on the diets studied, calculated by the following approximation (6), for Sample F. O. 1

$$\text{Per cent H.U.F.A.} = \frac{\text{polybromide No. lard esters} \times 100}{\text{polybromide No. esters of H.U.F.A.}} = \frac{2.40 \times 100}{89.3} = 2.7$$

is much less than found for rats on similar diets for reasons already suggested. The results differ further from those on rats in that the deposited acids were in this instance less unsaturated, whereas with rats they were more unsaturated, since the bromides from the rat fatty acids gave a higher bromine content than those from the oil-fed animals. The metabolic processes of the pig apparently select from the mixture of the unsaturated acids in the fish oil acids of average molecular weight but of lower iodine number for storage in the body fat. Or, explaining what happens from another view-point, the pig burns the more highly unsaturated acids from the oil, depositing the remainder. The writer believes that the metabolisms of the pig and the rat are sufficiently different to explain the apparent contradiction of results.

SUMMARY

1. When pigs were fed a diet containing about 14 per cent of menhaden oil, the lipids and fatty acids of the livers were decidedly more unsaturated than those of the controls, indicating a mobilization of highly unsaturated acids.

2. Under these dietary conditions there was a storage of 2.7 per cent of highly unsaturated acids in the lard.

3. The highly unsaturated fatty acids deposited were of about the same molecular weight and of lower iodine number than the mixture of acids isolated in a similar manner from the original menhaden oil.

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ANTIOXIDANTS AND THE AUTOXIDATION OF FATS*

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The control of autoxidation reactions by means of antioxidants and prooxidants (promoters) is increasingly useful in the industries. Instances are too numerous to mention. Investigations in the oil and rubber industries, in particular, have revealed a great variety of substances that may be employed to modify reaction velocity or to prevent deterioration of the product through oxidation or polymerization. Moureu and Dufraisse (1) were the first to propose an inclusive theory which should account for the effectiveness of almost infinitesimally small amounts of material in retarding or accelerating the processes of oxidation. In a recent review of autoxidation, Milas (2) has proposed an electronic explanation for these chain reactions in which molecular oxygen, autoxidizable materials, accelerators, and inhibitors take part. These reactions are characterized by a latent or induction period followed by active oxidation at the gradually increasing rate of an autocatalytic reaction.

The observations reported in this paper were made with the hope of securing some understanding of the nature and rôle of antioxygenic substances in edible fats. The production of sterility in rats by rearing them on synthetic rations containing butter fat and lard or cod liver oil (3) or on milk powder diets containing these unsaturated animal fats (4) was presumptive evidence that butter fat contained little vitamin E. The incorrectness of this idea was demonstrated by the reproductive adequacy of milk powder rations not containing lard or cod liver oil, by the destruc-

* Reported in part before The Thirteenth International Physiological Congress, at Boston, August, 1929 (Mattill, H. A., and Mattill, H. I., *Am. J. Physiol.*, **90**, 447 (1929)).

tive effect of these and certain other fats upon vitamin E (5), and by the antioxygenic effect of wheat germ oil upon the autoxidation of these fats (6).   The case for autoxidative destruction was further strengthened by the relation of oxidation to the vitamin E content of milk powders (7) and by observations that iron salts shortened the induction period (6) and hastened the disappearance of vitamins A and E from rations which originally possessed them (8–11).   Additional evidence comes from the fair degree of parallelism between the ease of autoxidation of fat mixtures and the reproductive performance of rats on diets containing them (12).

Aside from its content of vitamin E, therefore, wheat germ oil possesses a constituent which has antioxygenic power.   Corn oil also contains such a substance (13) and the superior keeping qualities of vegetable oils as compared with animal fats of equal or even less unsaturation may be ascribed to the presence of these oxidation inhibitors.   They are found in the non-saponifiable portion of the oils (13) and work on their isolation and further study is in progress.

Certain hydroxyl derivatives of aromatic compounds have long been known as inhibitors of oxidation and inasmuch as the sterols are hydroxy aromatic derivatives some information as to the nature and structure of the antioxidizing sterols should be gained from a study of the behavior of hydroxy aromatic compounds of known constitution.   The relation of inhibitor to autoxidant is complex and in these studies the latter has been limited to lard, cod liver oil, or a mixture of them; the fact that a given substance may be useful as an antioxidant in one connection is not a guarantee of its universal applicability.

EXPERIMENTAL

Since the effect of an antioxidant is confined largely to the latent or induction period, before active absorption of oxygen has begun, some means of measuring this interval, without reference to the further progress of oxygen absorption, sufficed for these tests. The apparatus used was a slight modification of that described by Greenbank and Holm (14), for determining the thermal oxidation induction period.   Erlenmeyer flasks containing 5 or 10 gm. of the fat were immersed in a calcium chloride solution maintained at 70°.   The flasks were connected to open manometers through a

mercury seal, the air in the flasks being displaced by oxygen in a slow stream through a side tube. Platinum contacts in the manometers were connected to signal magnets writing on a slow kymograph, the upper of the two platinum wires in a manometer being just above the surface of the mercury. By means of half-hourly contacts from a clock the first diminution in volume due to absorption of oxygen was thus recorded. The further progress of oxygen absorption could be observed in the continued rise of the mercury column, but as Milas has pointed out (15) too many variables are involved to make this a satisfactory quantitative method. Some of his objections may apply even to the measurement of the induction period, notably the possibility of the formation of a surface film, since the mixture was not stirred. However in these fats polymerization is unlikely at 70°. The oxygen was not washed nor were any measures taken to remove water from the fats; more uniform results could be expected if the fats were not dehydrated since water retards autoxidation.

As indicated elsewhere (12) there is a great and almost mysterious variability in the induction periods of different samples of the same fat, due probably to differences in origin and conditions of manufacture and storage, and it seemed necessary to carry a blank determination with each trial of antioxidants. With proper attention to cleanliness of glassware and uniformity of procedure it was possible to obtain satisfactory repetition of the blank determinations. The induction period of most samples of cod liver oil seemed too short to be accurate under the conditions of measurement; that of good lard is too long for convenience and changes in atmospheric pressure require too frequent correction on the manometers. Such changes have often been disturbing even in the shorter periods. The most satisfactory fat mixture seemed to be 5 gm. of lard and 10 drops (0.32 gm.) of cod liver oil and this mixture has the further advantage of being one of those commonly used in rat feeding experiments. For the usual lard and cod liver oil samples the blank induction periods varied from 6 to 10 hours. Doubtless other methods are available for measuring the induction period of fat oxidation. The successful use of methylene blue (16) in photoelectric work suggests its applicability in thermal studies. It is not claimed that the present procedure has the merits of a rigorous quantitative method. With

certain modifications its accuracy can be increased but for purposes of orientation it is simple and reliable. Reasonable agreement between two to five and often more tests was always secured before the results were accepted.

The substances to be tested as antioxidants were used in the quantity of 0.02 per cent of the fat mixture. A few of them were sufficiently insoluble in fat that even this small amount could not be taken up completely. The induction period (in hours) with antioxidant divided by the induction period of the blank is designated as the antioxygenic index and Table I contains this index for the substances that have been examined. A figure of 0.8 to 1.3 for this index has been interpreted to mean that the substance has no antioxygenic effect, that is, the difference in induction periods of the fat with and without the added material is too small to be significant. These inactive compounds have been listed separately from the active ones.

Only a few of the substances examined possess the capacity to retard or inhibit the autoxidation of fat. Phenol was ineffective even when 2 per cent was used; m-cresol was likewise inactive and the other cresols and thymol were only very slightly active. Of the dihydroxy derivatives of benzene the ortho and para compounds were very powerful, the meta extremely feeble. When the ortho compound contained OCH_3 in place of one of the hydroxyls (guaiacol) it was only slightly better than the meta compound. Of the trihydroxy benzenes examined, the symmetrical phloroglucin acted feebly, pyrogallol powerfully. That the hydroxyl group is responsible for antioxygenic action (13) was verified by the inertness of hydroquinone diacetate. Since p-oxybenzoic and salicylic acids were both inert, the two hydroxyl groups must be directly bound to the ring. The fully hydroxylated benzol compound, inosite, was inactive.

Of the substances containing two or more benzene rings, α-naphthol was more than ten times as effective as the β derivative; β-naphthoquinone was very efficient while the α derivative was inert.

Two very recent papers by Tanaka and Nakamura (17) contain data on the iodine number of linseed oil after exposure to oxygen with and without antioxidants. They indicate the superiority of α- to β-naphthol and the order of effectiveness of the

di- and triphenols is essentially that found in this study. Moureu and Dufraisse (18) found phenol and resorcinol to have very feeble action in connection with a great variety of autoxidizable substances other than unsaturated fatty acids, while pyrocatechol, hydroquinone, and pyrogallol were very effective. Others (19), still earlier, had made like observations. A number of more recent papers (20–25) contain similar information, most of it in agreement with the facts outlined in Table I. Variations in the relative amounts of antioxidant used may explain some of the apparent contradictions. Moureu and Dufraisse (26) state that anti-oxidation is proportional to the amount of antioxidant present but the proportionality may not be uniform for all antioxidants.

Although the relative activity of various phenols as antioxidants has thus been generally known for some time, apparently no one has yet pointed out that this activity is in some manner associated with the ortho, para configuration and is not possessed by compounds with the meta configuration or its equivalent.[1] The reaction chains which are started when an unsaturated fat takes on oxygen can be broken by the former but not by the latter type of phenols. The course of oxidation, through "moloxides," peroxides, and ozonides (27) is interrupted by the preferential oxidation of minute amounts of the more readily oxidizable phenols but this explanation becomes less satisfying when one seeks farther.

Since to the physical chemist "it seems at present impossible to give very precise definition to the ideas which we express in the term unsaturation" (28) and since the structure of the benzene ring is still one of the live issues among organic chemists, an extended attempt to explain the observations made would likely be profitless, if not out of place, even if the writer were competent to make it. A few comments may be permissible. Most theories of autoxidation assume that a very few of the molecules of the autoxidant are in a much more reactive form than the rest. In

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[1] Seyewetz and Sisley (*Bull. Soc. chim.*, **31**, 672 (1922)) in calling attention to the origin of the term antioxidant which was first proposed by Lumière and Seyewetz (19), also point out that they (19) associated anti-oxygenic properties with the capacity to act as a photographic developer. The commonly used developers are easily oxidizable ortho and para derivatives.

the recent valuable discussion mentioned above (2) Milas has made the assumption that "in all auto-oxidations . . . the atoms to which the oxygen molecule initially adds may be regarded as making definite contributions of two electrons to it," forming metastable or "dative" peroxides of high instability and energy content. Furthermore, the possession of unshared or "exposed" electrons, is also a characteristic of inhibitors and "if an inhibitor collided with the dative peroxide, all the excess energy of the latter would be completely absorbed by the molecular valence electrons of the former, thus the initiation of new reaction chains is prevented. . ." In his study of electron displacement in the benzene ring Lucas (29), employing the Pauling structure of benzene and its derivatives, has demonstrated that the introduction of a positive group (like OH) makes the p-hydrogen most negative, o- next, and m- least negative. Since phenol, as shown above, is not an antioxidant, we may assume that the electrons even though displaced away from the ring have not been displaced far enough. Lucas does not discuss the effect of the introduction of a second (OH) group in the ortho or para position but this should increase the electron displacement still more, while the influence of such a group in the meta position would tend to be dissipated. If the theory of electron displacement in the ring contains the explanation of antioxygenic activity it should be possible to predict the effectiveness of antioxidants (various OH, NH_2, Cl, and alkyl derivatives) and conversely the antioxygenic capacity of compounds might throw some light on their electronic structure.

The antioxygenic capacities of α- and β-naphthol indicate that the former has the character of an ortho compound, the latter that of a meta, a fact which their differences in reactivity have long indicated. A recent excellent review by Obermiller (30) summarizes the varied evidence. It is interesting that α-naphthol is so effective an antioxidant although containing only one hydroxyl group. The introduction of a methyl group into phenol in the ortho position (o-cresol) increased the antioxygenic capacity only very slightly; the presence of another benzene ring joined as it is in this position is much more effective.

The behavior of the quinones is not to be explained at present; it is surprizing that any of them should have antioxygenic activity.

Nor is it evident why quinone and α-naphthoquinone should act so differently. A peculiarity in the action of quinone should be mentioned. The rate of oxygen absorption, once oxidation has begun, is ordinarily the same, whether an inhibitor is present or not; Yamaguchi (31) has recently reported a similar observation. In the case of quinone a slow absorption of oxygen proceeded for some time before the autocatalytic character of the reaction was manifested. If this behavior indicates the presence of traces of hydroquinone in equilibrium with quinone a similar condition does not exist in the naphthol derivative. The recognized stability of anthraquinone and its derivatives receives further evidence in the lack of antioxygenic activity.

Gossypol and its derivatives (32) were of interest because of the toxic action and the presence of six hydroxyl groups. It is again evident that the OH group is concerned with antioxygenic capacity. In view of the relatively harmless character of hydroquinone when ingested, the toxicity of gossypol can hardly be related to its feeble activity as an inhibitor of oxidation.

The various sterol preparations of animal origin and sitosterol from three different plant sources were all of them inactive. This was to be expected since there is only one hydroxyl, although the presence of some other sufficiently positive group might serve in its place. The antioxygenic sterols of vegetable fats are not precipitable by digitonin and methods for their separation are greatly needed. Estill and McCollum (33) obtained from cod liver oil a compound with LiCl which was evidently antioxygenic since it prevented "salt ophthalmia" in rats on diets containing ferrous sulfate. The fact that a substance with this property could be obtained from a readily autoxidizable animal fat is significant. Perhaps oxidation in the animal body is in part governed by the presence of traces of inhibiting substances of this kind. Epinephrine is an o-dihydroxy compound, thyroxine is allied to a p-dihydroxy compound.

In vitro experiments (34) have indicated that carotene is a prooxidant, and its greater effectiveness as vitamin A when administered in peanut oil as compared with petroleum oil (35) or ethyl oleate (36, 37) is very reasonably explained by the presence of antioxidants in vegetable oils. When this natural protection is not afforded some other inhibitor must be present if the vitamin is

to survive even a short period.   In the preparation and manipulation of other readily oxidizable materials like phospholipids the addition of traces of an antioxidant would greatly retard undesirable oxidative changes.

The production of prooxidants by the irradiation of fats with ultra-violet light is well recognized.   Irradiation of the mixed sterols of corn oil (13) produced traces of peroxide substance and

TABLE I

Antioxygenic Index* of Substances toward Lard and Cod Liver Oil

Ineffective substances, index of 0.8–1.3	
Phenol, *m*-cresol	*p*-Cresol, 1.4–1.7
p-Oxybenzoic acid, *p*-oxybenzaldehyde	*o*-Cresol, 1.2–1.4
Salicylic acid, salol	Thymol, 1.2–1.7
Inosite,† hydroquinone diacetate†	Hydroquinone, 120
Phenolphthalein, phthalic acid	Pyrocatechol, >55
α-Naphthoquinone†	Guaiacol, 1.4–1.8
Anthraquinone, 1,5-dihydroxy anthraquinone,† alizarin, ‡ purpurin	Resorcinol, 1.3–1.7
	Orcinol, 3–5
	Quinone, † 8–10
Hexacetyl gossypol,§ hexacetyl apogossypol§	Pyrogallol, >64
	Phloroglucinol, 2.8–3.3
Cholesterol,‖ ergosterol,†,‖ sitosterols, ¶ acterol, oscodal,** non-saponifiable matter of cod liver oil (specially prepared)	α-Naphthol, 31
	β-Naphthol, 1.3–1.9
	β-Naphthoquinone, † 23–27
	Gossypol, § 3–5

* This index is the ratio of the induction period (in hours) with antioxidant to the induction period of the fat with no addition.

† Eastman Kodak Company.

‡ National Aniline and Chemical Company, Incorporated.

§ Kindness of Dr. E. P. Clark.

‖ Both before and after irradiation.

¶ Prepared from corn, wheat, and lettuce.

** Kindness of Dr. Dubin.

recent literature contains several suggestions that sterols are oxidative catalysts especially after irradiation, but in the quantity (0.02 per cent) used in these tests, cholesterol and ergosterol behaved alike both before and after exposure to ultra-violet light.

The probability that the functions of fat-soluble accessories are not comprised within the commonly known effects of vitamins A, D, and E or that they are not yet properly allocated, is suggested by several observations.   Most recent of these (38) is the appear-

ance of muscular dystrophy in rabbits on vitamin E-free diets. The striking discovery that vitamin A rather than vitamin E is concerned with sterility in the male rat (39) we have recently confirmed. The growth-promoting function of vitamin E (40, 41) has not been explained nor has the effectiveness of butter fat in the cure of "black tongue" in dogs (42). Progress in the solution of these problems depends on the successful segregation of the factors concerned, among them probably pro- and antioxidants.

SUMMARY

To secure information on the chemical nature of the antioxygenic substances that are found in natural oils and that prevent the autoxidative destruction of fat-soluble vitamins a series of hydroxy aromatic compounds was tested for their capacity, when used in a quantity of 0.02 per cent, to prolong the thermal oxidation induction period of a standard mixture of lard and cod liver oil. The observations indicate that antioxygenic capacity of phenols resides in two hydroxyl groups in the ortho or para configuration; when these are in the meta position the compound is inactive. The hydroxyls are ineffective unless attached directly to the ring; the fully hydroxylated inosite is inactive. In the naphthols one hydroxyl group is sufficient and in keeping with its accepted behavior, α-naphthol has the character of an ortho compound and is much more effective, as an antioxidant, than β-naphthol; quinone is effective and β-naphthoquinone is even more so but the α form is entirely inactive.

The relation of these facts to the more recent theories of the electronic structure of the benzene ring and autoxidation is briefly discussed and it is suggested that in the preparation and manipulation of easily autoxidizable substances, the presence of traces of antioxidant will prevent undesirable oxidative changes.

A number of sterols of animal origin and sitosterol from wheat, corn, and lettuce were all inactive.

The existence of pro- and antioxygenic substances among the non-saponifiable constituents of natural fats and oils suggests that some of these may be concerned with the physiological action of the fat-soluble vitamins, and methods for their segregation from the sterols are under investigation.

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A STUDY OF THE EFFECT OF NITROUS ACID UPON COMPONENTS OF THE VITAMIN B COMPLEX*

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In much of the work which has been done in attempts at isolation, vitamin B has been precipitated under conditions suggesting that its chemical nature might be that of a nitrogenous base. Funk (1), Edie, Evans, *et al.* (2), Drummond (3), and Kinnersley and Peters (4), using extracts of rice polishings or yeast, obtained precipitates with phosphotungstic acid and with heavy metal salts which showed the presence of the antineuritic substance. Seidell (5) prepared an insoluble picrate from yeast which cured polyneuritis in fowls. Drummond and Funk (6), using a commercial yeast preparation obtained a precipitate with phosphotungstic acid, which, when decomposed to remove the acid, showed the presence in small amount of a growth-promoting substance when fed to rats on an otherwise vitamin B-free diet.

This work antedated the differentiation of the vitamin B complex; but as most of the tests for the vitamin were made by prevention or cure of polyneuritis in birds, it may be considered as dealing with vitamin B in the present sense.

Several investigators have undertaken to determine whether or not vitamin B is of amine nature by subjecting it to treatment with nitrous acid. The results, however, have been variable. McCollum and Simmonds (7) treated an alcohol extract of wheat embryo by aspirating through it the gases from a nitrous acid generator. The solution was then fed to rats which had reached a stationary weight or were declining on a vitamin B-free diet. From their results they concluded that it was possible that treatment with nitrous acid tended to destroy vitamin B to a slight extent

but very slowly and that this resistance was a strong indication that the vitamin was neither a primary nor a secondary amine.

Peters (8) used an acid yeast concentrate and by addition of saturated sodium nitrite generated nitrous acid in the solution. This yeast solution when fed to polyneuritic pigeons showed no appreciable destruction of the curative substance. Hence he concluded that the vitamin is probably not a primary amine, but pointed out that other nitrogenous bases including some containing the :NH group might remain unchanged under the treatment which he had applied.

In 1926, Levene and van der Hoeven (9) mention deaminizing their vitamin B fraction from yeast by nitrous acid. From this deaminized fraction they reported the adsorption of the vitamin on silica gel. Later, 1928, Levene (10) states that a longer period of observation showed the fraction was ineffective for growth. He further states that by means of nitrous acid the activity of the heat-stable factor (vitamin B_2 or G) is destroyed but the heat-labile factor (vitamin B or B_1) remains.

Still more recently, however, Chick (11) has found vitamin B_2 (G) to remain apparently unaffected under nitrous acid treatment which reduced to one-sixth the amino nitrogen present, and Narayanan and Drummond (12) have also found no appreciable destruction of vitamin B_2 to result from the action of nitrous acid. In the latter experiments 100 cc. of the vitamin solution containing 1.5 gm. of organic matter were treated with 2.5 gm. of sodium nitrite and the mixture cooled on ice. Then 10 per cent hydrochloric acid was added and the mixture allowed to stand for 10 minutes. The solution was then warmed to 60–70° and finally neutralized with sodium hydroxide solution.

Before the publication of these latter investigations and without knowledge of them, the experiments reported in the present paper were performed with the purpose of throwing further light upon the differentiation of vitamins B (B_1) and G (B_2) by means of strictly parallel measurements of the effects of nitrous acid upon these two vitamin values or potencies in the same solution. As a suitable material containing both of these vitamins we chose the well known "protein-free milk" preparation of Osborne and Mendel (13). Our work may be regarded as extending that of previous investigators, first, by the use of different nitrous acid treatments

including a more drastic form of treatment, and second, by the strictly parallel study of the two vitamin potencies in the same material before and after treatment.   In addition we have sought to interpret the experimental data in the light of the present evidence that more than two components of the vitamin B complex may be involved.   Thus we hope that the present paper may help to clarify the apparently conflicting findings of previous investigations.

<div align="center">EXPERIMENTAL</div>

Preparation of Protein-Free Milk—To avoid possible variations in results due to the use of different samples of milk at different seasons, a quantity of skimmed milk powder sufficiently large for the experiments was made into one composite lot.   To prevent unnecessary dilution in the preparation of the protein-free milk the following plan was adopted: 500 gm. of skimmed milk powder were mixed with 4000 cc. of distilled water, 890 cc. of 1 per cent hydrochloric acid[1] were stirred in, and the coagulated casein was removed by pouring the mixture through cheese-cloth.   The filtrate was boiled 5 minutes to precipitate the lactalbumin and was then filtered.   The filtrate was neutralized to pH 5.7 by 0.2 M sodium hydroxide.

Treatment with Nitrous Acid—The solution was treated with nitrous acid in two ways.

1. Nitrous acid was generated by the action of sodium nitrite and hydrochloric acid and the gases from the generator were drawn by aspiration through sodium nitrite solution and then into the vitamin solution.   For 500 cc. of the unneutralized protein-free milk, pH 4.2, which corresponds to 70 gm. of skimmed milk powder, the gases from 100 gm. of sodium nitrite and 1:1 hydrochloric acid were passed through for 4 hours.   The solution stood at room temperature 14 hours.   After this time the dissolved gases were removed under reduced pressure (30 mm.) at about 35–40°.   The liquid was then neutralized with 0.2 M sodium hydroxide.

2. Due to the instability of nitrous acid, the above treatment might be insufficient.   For this reason a more drastic method of

[1] The 1 per cent hydrochloric acid was prepared by diluting 28 cc. of concentrated hydrochloric acid (sp. gr. 1.18) to 1000 cc.

treatment was adopted.   In 500 cc. of the acid protein-free milk filtrate, pH 4.2, 25 gm. of sodium nitrite were dissolved and the stoichiometrical quantity of 1:1 hydrochloric acid was added drop by drop through a dropping funnel reaching to the bottom of the liquid.   The solution stood 14 hours at room temperature. It was then heated at 35° and 30 mm. pressure till all foaming ceased and was neutralized as described above.   All solutions were prepared each week.

Methods of Measuring the Vitamin B and G Values

In order to learn whether or not the vitamins were affected by the nitrous acid, the vitamin B and vitamin G potencies of all solutions were determined quantitatively before and after treatment. The later developments of research in several laboratories including our own make it now probable that more than one substance may be involved in each of these potencies.   This adds to the complexity of the problem, and thereby to the significance of the results, especially as helping to harmonize the previous findings with each other and with those here reported.

The rat growth methods for determination of vitamin B as worked out by Chase (14) and for determination of vitamin G as worked out by Bourquin (15) were followed.   The material to be tested was fed to rats receiving the vitamin B- or vitamin G-deficient diets.   The growth produced in rats by the solutions treated with nitrous acid was compared with that produced by the untreated vitamin solution when fed in corresponding amounts.

Effect of Hydrochloric Acid 1:1 Concentration

When the vitamin solution was treated by adding directly to it the sodium nitrite and 1:1 hydrochloric acid, the vitamin-containing material was left for some hours in contact with a very high concentration of hydrogen ion, pH 0.20 to 0.17.   Although the indications are that the water-soluble vitamins are acid-stable, it seemed advisable to determine whether or not any vitamin destruction could be attributed to the hydrochloric acid thus used in generating the nitrous acid.   A portion of the vitamin solution was treated in the same manner as that in which the nitrous acid was made with the exception that no sodium nitrite was added. To 576 cc. of protein-free milk, pH 4.2, corresponding to 72 gm. of

skimmed milk powder, 81 cc. of 1:1 hydrochloric acid (equal volumes of hydrochloric acid, sp. gr. 1.18 and water) were added and the mixture was allowed to stand at room temperature for 14 hours. It was then neutralized with 0.2 M sodium hydroxide to pH 5.7. The volume was reduced by vacuum distillation at 30 mm. and at 40°. It was fed in amounts corresponding to 0.9 gm. of skimmed milk powder with the vitamin B-deficient diet and corresponding to 0.4 gm. of skimmed milk powder with the vitamin G-deficient diet.

The animals receiving the hydrochloric acid-treated solution plus the vitamin B-deficient or vitamin G-deficient basal diets made gains essentially the same as their litter mates which received corresponding amounts of the untreated vitamin solution with the same basal diets. This would indicate that neither the vitamin B nor vitamin G concentration of this solution was measurably diminished by standing 14 hours at room temperature acidulated with hydrochloric acid as here described.

Effect of Nitrous Acid on Vitamin B (B_1)

When the solutions treated with nitrous acid by aspiration and by generating the acid directly in the liquid were fed, the material which had been treated directly with nitrous acid was not readily taken by the animals. In some instances it was eaten only fairly well. Two additional experiments were undertaken in an effort to obtain evidence based on complete consumption of the supplements: One consisted in hand feeding the daily portions; in the other, the nitrous acid treatment was made one-fourth as drastic as had been used.

Results—Seven animals receiving in addition to a diet deficient in vitamin B (B_1) the vitamin solution treated with nitrous acid by aspiration made an average gain during the 8 weeks period of 19 gm. They lived and seemed vigorous throughout the period. Six animals voluntarily taking the vitamin solution directly treated with nitrous acid showed an average *loss* of 22.5 gm. Their days of survival ranged from 19 to 50, an average of 35. Six animals were given the directly treated solution by hand feeding. Their average loss was 30 gm. and their survival period averaged 22 days. Five animals receiving the milk treated with one-fourth the amount of nitrous acid showed an average loss of 0.2 gm.

They lived throughout the period.   One developed polyneuritis in 10 weeks.   Sixteen negative controls showed a loss of 28.3 $\pm$ 1.3 gm. with an average survival period of 24.3 days.   Ten positive controls receiving the untreated vitamin solution made an average gain during the 8 weeks of 28.3 $\pm$ 2.6 gm.

The animals receiving the solution treated by aspiration seemed vigorous and healthy to the end of the period.   But those fed the directly treated solution showed the typical symptoms of vitamin B (B_1) deficiency.

As a safeguard of interpretation we have also made separate comparison of the averages obtained in those cases in which the directly parallel tests were made upon litter mates.   We have taken account of the gains shown in 4 week as well as 8 week experimental periods, since the second 4 weeks is more likely than the first to be influenced by other factors.   As these results are essentially confirmatory of those just given the details may be omitted to save space.

Our findings indicate that treatment by the aspiration method had little if any influence upon vitamin B (B_1), whereas the drastic treatment with nitrous acid generated very abundantly directly in the vitamin solution resulted in a large diminution of the vitamin B (B_1) potency as shown both by the weight curves of the experimental animals and the development of polyneuritic symptoms.

The experiments with a less drastic direct treatment yielded an intermediate result.

From these results and those of other workers already mentioned one might logically conclude that vitamin B (B_1) is susceptible to the action of nitrous acid, but only under more drastic conditions of treatment than are usually employed.   This suggests that vitamin B may be a nitrogenous base, but of such structure as to be more resistant to nitrous acid than are the typical primary amines.

Effect of Nitrous Acid on Vitamin G (B_2)

The vitamin solution treated directly and by aspiration with nitrous acid was fed to rats on the vitamin G-deficient diet (Diet 555).   Negative controls received the basal diet only, positive controls received, in addition, the untreated vitamin solution.

The results here obtained showed no destruction of vitamin G

(B_2) under treatment by the aspiration method. When the nitrous acid was generated directly in the vitamin solution the results of subsequent feeding tests were such as to indicate a partial destruction of vitamin G when the data are taken for an 8 week, but not when they are taken for a 4 week, experimental feeding period.   In the light of a number of recent suggestions of hitherto unknown factors in the vitamin B complex, and unpublished observations in this laboratory, we now interpret these findings as probable evidence that vitamin G (G_1, B_2) is itself stable toward nitrous acid, as Chick and also Narayanan and Drummond have found, and that certain indications of loss of vitamin G potency as in the work of Levene and in certain parts of our own work may perhaps better be considered as indicating more or less destruction of one of the newer and not yet clearly defined factors which also are now being found to be essential to the growth of rats, and which as the experience of this laboratory in other connections indicates, may sometimes complicate the measurements of vitamin G potencies, especially when the material under investigation is a laboratory fraction rather than the entire vitamin G complex of a natural food material.

There are also indications that, whatever the component of the vitamin G complex which is affected by the nitrous acid treatment, the reaction involved may be one of oxidation rather than deaminization in this case.   The losses of potency incurred in evaporating and drying vitamin-containing solutions upon starch, as well as the results of certain experiments with ozone, seem to show that, while even less susceptible than vitamin B to nitrous acid, vitamin G (or some component of the vitamin G complex) is more susceptible to oxidation.

Our results as a whole appear to be in harmony with the view of Salmon and his coworkers (16) that vitamin B behaves *in vitro* more like a nitrogenous base and vitamin G more like a neutral organic substance.

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A SIMPLE CONTINUOUS DIALYZER

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A common operation, particularly in bacteriological chemistry, is the removal by dialysis against distilled water of ammonium sulfate or other precipitant from small preparations of protein. Occasionally, too, it is necessary to determine the amount of an inorganic compound present in a protein substance, for example the amount of iodide in an impure iodoprotein. Determinations of this nature can often be conveniently carried out by dialysis, most quantitatively when a continuous dialyzer is employed. An account of a simple continuous dialyzer which has proved very satisfactory for such purposes is given below.

The construction of the dialyzer is shown in Fig. 1, which represents a small scale apparatus suitable for volumes of about 10 to 15 cc. The flask A contains distilled water, or dilute acid or alkali, which is kept boiling steadily by means of the burner, a small fragment of pumice stone being added to prevent bumping. A sheet of asbestos, S, is arranged to shield the dialyzer from the heat of the flame. The steam passes up the tube B (which is lagged with cotton wool) and is condensed in C. Most efficient cooling is obtained with a condenser of the type shown. The tube B has an internal diameter of about 3 mm. and a length of about 45 cm. It is made fast to the condenser with rubber bands as at D. E and E' are slices of cork. The water condensed in C passes down the condenser and accumulates in F until it actuates the siphon G, runs into the dialyzing system in H, and displaces the dialysate via the siphon I into the flask A. This process is continuous and takes place several times in an hour.

An arrangement of the dialyzer H which is attached to the tube G by means of a rubber joint is shown in detail in Fig. 2. The essential point is that the solution to be dialyzed is not placed

in the parchment sac (shown by the heavy line), but in the outer vessel H. The mouth of the sac, which in the apparatus shown is of the ordinary type about 5 inches long and $\frac{3}{4}$ inch in diameter, is closed by means of the rubber stopper J to which it is firmly
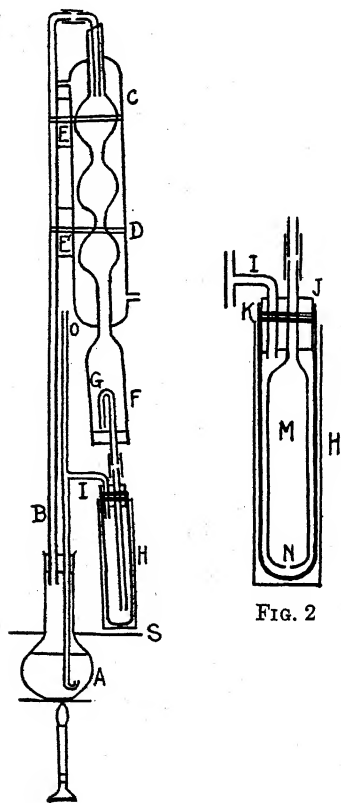
FIG. 1

FIG. 1. A simple continuous dialyzer
FIG. 2. Arrangement of the dialyzer

attached by many turns of a small rubber band K. The water from the condenser passes into the tube M, out at the opening N, and after traversing the inner surface of the sac returns to the flask A through the siphon I. Thus the diffusible electrolyte accumulates in the flask A and can be readily estimated when dialysis

is complete. The tube M is inserted to decrease the effective volume of the contents of the sac and to insure circulation of the entering water. The tube is made by drawing out a closed tube of suitable diameter and making a small hole N at the bottom. The siphon I is made from tubing of 2 mm. internal diameter. The purpose of the open vertical tube O is to prevent air being drawn into the dialyzer after siphoning takes place, and to enable the dialysate to be sampled at any stage. This is done, if required, by attaching a small piece of rubber tubing to the upper end of O and, after removing the burner, drawing off some of the contents of the sac through O by means of a pipette.

It will be seen that a relatively small amount of the solution to be dialyzed is made to cover a large surface of membrane and the process of dialysis is correspondingly accelerated. The efficacy of the parchment sacs which are generally very durable can be tested at any time by dialyzing a mixture containing starch solution and potassium iodide and testing for starch in the dialysate. A new sac can be readily attached by placing it, after soaking it in water, in the vessel H, round the upper lip of which is wound a number of turns of a rubber band. The cork is inserted into the sac and the rubber band is slid off the lip of the tube whereupon it snaps into place, binding the parchment firmly to the cork.

THE APPARENT DISSOCIATION CONSTANTS OF PHENYL-ALANINE AND OF DIHYDROXYPHENYLALANINE AND THE APPARENT FREE ENERGY AND ENTROPY CHANGES OF CERTAIN AMINO ACIDS DUE TO IONIZATION*

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The present work is a continuation of the systematic survey of the apparent dissociation constants of the amino acids which has been in progress in this laboratory for some time.[1] In this paper we are reporting not only the apparent dissociation constants of phenylalanine and 3,4-dihydroxyphenylalanine but also the apparent change in free energy, $\Delta F^{o\prime}$, and the apparent entropy change, $\Delta S'$, at 25° of such naturally occurring amino acids for which data upon which to base calculations are available.

The method of obtaining the titration curves was the same as that which has been described by Kirk and Schmidt (1). The *dl*-phenylalanine was an Eastman Kodak product. It was re-crystallized three times. For the synthesis of *dl*-3,4-dihydroxy-phenylalanine, the procedure described by Hirai (2) was followed, except that, after the decomposition of the lead salt, our product was kept in an atmosphere of nitrogen rather than hydrogen and carbon dioxide, the technique which was employed by Hirai (2) and by Miller (3). Both products gave theoretical nitrogen values. It is noteworthy that on treatment with nitrous acid more nitrogen than the theoretical is obtained from 3,4-dihydroxy-phenylalanine. This is probably due to the setting free of nitro-gen from nitrous acid by the dihydroxyphenylalanine which acts

[1] See list of references given in Table I.

as a reducing agent.   Schmidt (4) has shown that on prolonged treatment with nitrous acid tyrosine yields more than the expected amount of nitrogen.

Fig. 1 shows the titration curve of phenylalanine.   Its apparent dissociation constants at 25° are: $K'_a = 5.82 \times 10^{-10}$, $K'_b = 3.83$

TITRATION CURVES
OF PHENYLALANINE

x = Amino Acid Curve-
0.02 M Solution.
o = Water Blank Curve.
----- = Corrected Titration Curve.
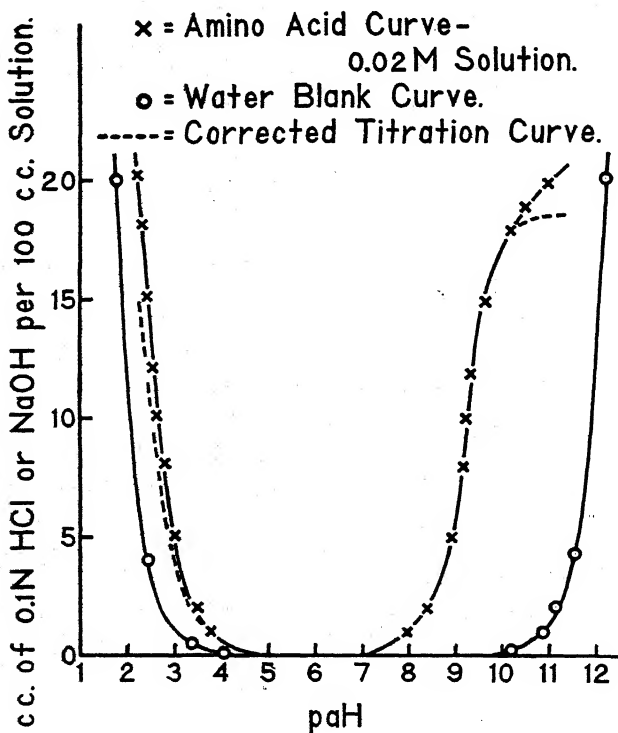
FIG. 1

$\times 10^{-12}$, and I $= 1.24 \times 10^{-6}$.   Our value for K'_a is in approximate agreement with that reported by Harris (5).   His value for K'_a is 7.5×10^{-10}.   Our value for K'_b agrees fairly well with that found by Kanitz (6).   His value for K'_b is 1.3×10^{-12} (7).

Fig. 2 shows the titration curve of 3,4-dihydroxyphenylalanine.

The values for the apparent dissociation constants at 25° are:
$K'_{a_1} = 2.11 \times 10^{-9}$, $K'_{a_2} = 1.33 \times 10^{-10}$, $K'_{a_3} = 2.10 \times 10^{-12}$, $K'_b = 2.31 \times 10^{-12}$, and $I = 3.12 \times 10^{-6}$.

TITRATION CURVES
OF 3,4-
DIHYDROXYPHENYLALANINE

x = Amino Acid Curve
       0.02 M Solution.
o = Water Blank Curve.
---- = Corrected Titration
                Curve.

Fig. 2

A comparison of the apparent acid dissociation constants respectively of alanine, valine, leucine, isoleucine, and phenylalanine (see Table I) shows that the magnitude of K'_a of phenylalanine is

somewhat greater than the constants of the first four mentioned amino acids. This appears to indicate that the effect of the phenyl ring in the methyl group of alanine increases the acid properties of the latter substance. A similar effect is shown by fatty acids in which a phenyl or other heavy cyclic group has been substituted. This view is supported by the fact that the magnitude of the values for the apparent acid dissociation constants of tryptophane and of histidine is approximately the same as that of phenylalanine. The presence of one or more hydroxyl or other negative groups in the phenyl ring markedly influences the acid properties of the amino acid. This is illustrated by tyrosine and 3,4-dihydroxyphenylalanine. Although we have no definite proof, it seems probable that the values of the largest magnitude for the apparent acid dissociation constants of tyrosine, 3,4-dihydroxyphenylalanine, and diiodotyrosine refer to the carboxyl group rather than to the hydroxyl group contained in the phenyl ring. The effect of the presence of the hydroxyphenyl ring in alanine is to increase the magnitude of the value for the apparent dissociation constant of the carboxyl group. The presence of iodine in the hydroxyphenyl ring of diiodotyrosine serves to increase its acid properties very markedly. It is also noteworthy that the magnitude of the values 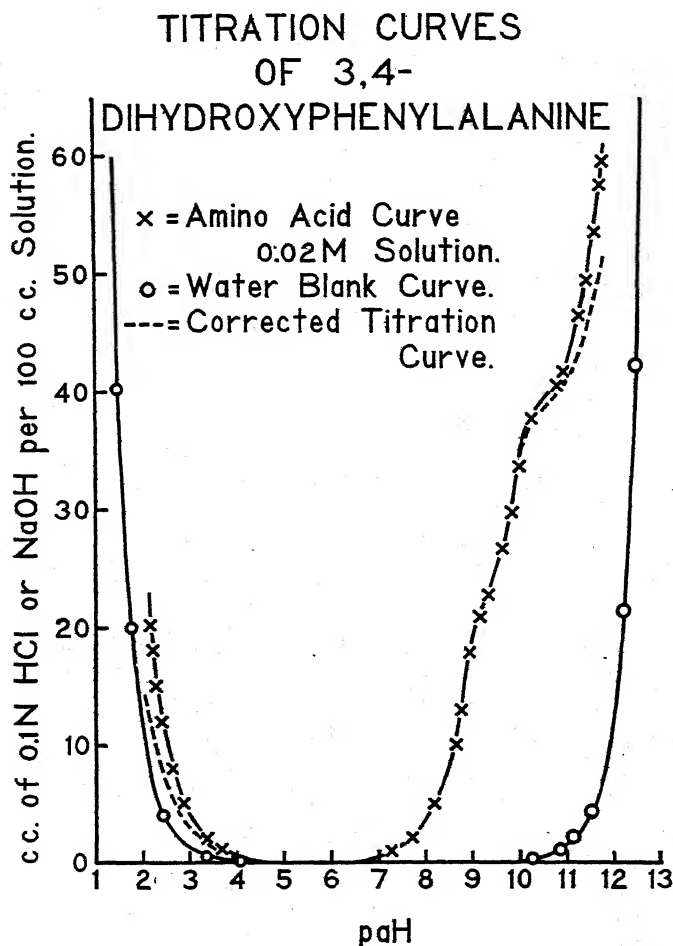for the apparent basic dissociation constants of alanine, leucine, glycine, isoleucine, norleucine, valine, phenylalanine, tyrosine, 3,4-dihydroxyphenylalanine, diiodotyrosine, and tryptophane is practically a constant.

The apparent free energy changes, ΔF, for the amino acids may be calculated from the values of the apparent dissociation constants of the amino acids as the following considerations show. Let us assume that a mol of each of A and B react to give a mol of each of C and D. Let a = activity, ΔF = the free energy change in this reaction when the substances are in any given states, and ΔF° = the free energy change when each substance is in its standard state.[2] Then

$$\Delta F - \Delta F^\circ = RT \ln \frac{a_C \, a_D}{a_A \, a_B} \tag{1}$$

_____

[2] ΔF° is defined for the present purposes as the free energy change when from an infinite volume of solution at 25° and a pressure of 1 atmosphere

At equilibrium $\Delta F = 0$.   Therefore

$$\Delta F^{\circ} = - RT \ln \frac{a_C\, a_D}{a_A\, a_B} \tag{2}$$

At any given temperature ΔF° is a constant.   The condition of equilibrium is such that the activity quotient shall also be constant.   The value of this quotient when the system is in equilibrium, we shall call the equilibrium constant, K, and write (8)

$$\Delta F^{\circ} = -RT \ln K \tag{3}$$

For the purpose of calculating ΔF° for the amino acids the true dissociation constants must be known.   For this purpose data for the activity coefficients are required.   Hoskins, Randall, and Schmidt (9) have determined the activity coefficients of aspartic and of glutamic acids at 0°.   Data for the activity coefficients of the amino acids at 25° are, however, not available.   In our calculations we have used the values for the apparent dissociation constants.   On the assumption that the relations expressed by equation (3) hold when the apparent values are used we may now write

$$(\Delta F^{\circ}{}_{298})' = -RT \ln K' \tag{4}$$

where $(\Delta F^{\circ})'$ and K' are apparent rather than absolute quantities.   The values for $(\Delta F^{\circ}{}_{298})'$ corresponding to the apparent acid and basic dissociation constants respectively of the naturally occurring amino acids for which data are available are given in Table I.

It is to be noted that the values for $(\Delta F^{\circ})'$ corresponding to K'_b of the monobasic amino acids and to K'_{b_2} of the dibasic amino acids are very nearly a constant.   The average value is 16,000.   The lower values for $(\Delta F^{\circ})'$ corresponding to K'_{b_1} of the diamino acids are a reflection of their respective larger primary dissociation constants.   The average value for $(\Delta F^{\circ})'$ corresponding to K'_a of the monobasic monoacidic amino acids is 13,000.   The value

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an amino acid at unit activity ionizes in accordance with one of the following equations

(1) $NH_2\, R\, COOH \rightleftharpoons \overset{+}{H} + NH_2\, \overset{-}{R}\, COO$

(2) $NH_2\, R\, COOH + HOH \rightleftharpoons NH_3\, R\, \overset{+}{C}OOH + O\overset{-}{H}$
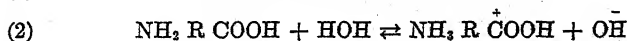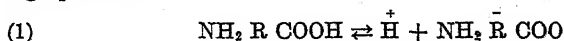
yielding ions which are also at unit activity.

TABLE I

Thermodynamic Data for Amino Acids at 25°

Amino acid	K'_a	K'_b	Bibliographic reference No.	$(\Delta F°)' K_a$ calories	$(\Delta F°)' K_b$ calories	$(\Delta H)' K_a$ calories	$(\Delta H)' K_b$ calories	Bibliographic reference No.	$(\Delta S)' K_a$ calories per degree	$(\Delta S)' K_b$ calories per degree
Monoacidic, monobasic										
Alanine	2.06×10^{-10}	2.21×10^{-12}	10	13200	15900	11450	14600	10	−5.9	−4.4
Cystine*	3.3×10^{-8}	5.0×10^{-13}	11	10200	16800					
	9.6×10^{-10}	1.5×10^{-13}		12300	17500					
Glycine	2.54×10^{-10}	2.21×10^{-12}	10	13100	15900	10700	14600	10	−8.1	−4.4
Isoleucine	2.09×10^{-10}	2.29×10^{-12}	1	13200	15900					
Leucine	2.5×10^{-10}	2.3×10^{-12}	5, 7	13100	15900					
Norleucine	1.72×10^{-10}	2.46×10^{-12}	1	13300	15900					
Oxyproline	1.86×10^{-10}	8.32×10^{-13}	1	13300	16500					
Phenylalanine	5.82×10^{-10}	3.83×10^{-12}		12600	15600					
Proline	2.5×10^{-11}	1.0×10^{-12}	12	14500	16400					
Serine	7.08×10^{-10}	1.62×10^{-10}	1	12500	16100					
Tryptophane	4.05×10^{-12}	2.4×10^{-12}	13	12800	15900					
Valine	2.4×10^{-10}	2.09×10^{-12}	1	13100	15900					
Monoacidic, dibasic										
Arginine	3.32×10^{-13}	1.10×10^{-5}	14	17000	6770	12400	2830	14	−15.5	−13.2
		1.05×10^{-12}			16400		10600			−19.3
Histidine	6.7×10^{-10}	1.01×10^{-8}	13	12500	10900	9400	7200	14	−10.5	−12.5
		6.6×10^{-13}			16600		12800			−12.8
Lysine	2.95×10^{-11}	0.89×10^{-5}	14	14400	6890	11600	1200	14	−9.3	−19.0
		1.52×10^{-12}			16100		13100			−10.2

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Monobasic, polyacidic Aspartic acid	2.24×10^{-4} 2.52×10^{-10}	†	7.51×10^{-13}	4980 13100	16500	1760 10500	10500	14	−10.8 −8.7	−20.2
Diiodotyrosine	3.32×10^{-7} 1.51×10^{-8}	15	1.32×10^{-12}	8840 10700	16200	810 8790	12770	15	−26.9 −6.3	−11.5
Dihydroxyphenylalanine	2.11×10^{-9} 1.33×10^{-10} 2.10×10^{-12}		2.31×10^{-12}	11800 13500 15900	15900					
Glutamic acid	5.62×10^{-5} 2.19×10^{-10}	1	1.55×10^{-12}	5800 13200	16100	1040 11200	12100	14	−16.0 −6.7	−13.5
β-Hydroxyglutamic acid	5.82×10^{-5} 2.76×10^{-10}	1	2.12×10^{-12}	5780 13100	15900					
Tyrosine	6.6×10^{-10} 4.9×10^{-11}	16	1.75×10^{-12}	12500 14100	16000					

* Cystine is a diacidic and dibasic amino acid. It is placed in the group of monoacidic, monobasic amino acids for convenience only.

† Calculated from the values for $\Delta H'$ at 25° (14).

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for $(\Delta F°)'$ corresponding to K'_a of arginine deviates considerably from this average value. This is due to the extremely weak acidic properties of this amino acid. The value for $(\Delta F°)'$ corresponding to K'_a of lysine and of histidine respectively fall within the range of the values for $(\Delta F°)'$ of the monoacidic monobasic amino acids. The low values for $(\Delta F°)'$ corresponding to the primary apparent acid dissociation constants of the polyacidic amino acids are a reflection of their stronger acidic properties. The values for $(\Delta F°)'$ corresponding to the K'_{a_2} of these amino acids do not deviate markedly from the average value of $(\Delta F°)'$ corresponding to K'_a of the monoacidic monobasic amino acids.

We have also calculated values for $\Delta S'$, the apparent change in entropy of the system due to ionization for such amino acids for which the values of $\Delta H'$ are known. The relation between free energy, heat of ionization, and entropy is expressed by the equation ((8) p. 169)

$$\Delta F - \Delta H = -T \Delta S \tag{5}$$

where $\Delta F =$ the free energy change, $\Delta H =$ the change in heat content of the solution as the result of ionization, $\Delta S =$ the entropy change of the system, and $T =$ the absolute temperature. In our calculations the assumption was made that the relation expressed by equation (5) holds when the apparent instead of the true values for ΔF, ΔH, and ΔS are used and this has been indicated by the prime mark after each of these terms.

It is not possible at the present time to calculate values for $\Delta S'$ for all of the amino acids since the values for $\Delta H'$ of only about one-third of the naturally occurring amino acids are known. However, the $\Delta H'$ values include members of each of the three groups, viz., monoacidic monobasic, hexone bases, and monobasic polyacidic amino acids. The $\Delta S'$ values are probably the least accurate of the three groups of thermodynamic data which are given in Table I. This is necessarily so since the values for $\Delta S'$ include errors in the values for both $\Delta H'$ and $(\Delta F°)'$.

In all of our calculations the $\Delta H'$ values are smaller than the corresponding $(\Delta F°)'$ values and consequently the ΔS values are negative. It is evident from the calculations that in the ionization heat is not absorbed but given up to the surroundings.

The data of Hoskins, Randall, and Schmidt (9) relating to the

activity coefficients of aspartic acid and of glutamic acid at 0° permit us to test the validity of the assumption that for practical purposes the values for the apparent primary dissociation constant of glutamic acid and of aspartic acid may be used in place of the true dissociation constant without introducing serious errors. In a solution of an amino acid such as glutamic acid or aspartic acid which contains not only the free amino acid but also some of its sodium salt, we have the following relations.

$$\mathrm{pK}_a = \mathrm{paH} + \log \frac{\textit{activity of undissociated amino acid}}{\textit{activity of dissociated amino acid}} \qquad (6)$$

where K_a = the true dissociation constant.

Activity of the undissociated amino acid = (concentration of amino acid −
        concentration of amino acid in form of ions) γ_u (7)

where γ_u = the activity coefficient of the undissociated amino acid.

The monosodium salt of aspartic acid and of glutamic acid is ionized in accordance with the equation

$$\mathrm{Na\,A} \rightleftarrows \overset{+}{\mathrm{Na}} + \overset{-}{\mathrm{A}} \qquad (8)$$

where $\bar{\mathrm{A}}$ represents the concentration of aspartate or of glutamate ion. The degree of ionization corresponding to the concentration of the monosodium salt of the amino acid was interpolated in each instance from the data of Hoskins, Randall, and Schmidt (9).

Since free glutamic acid is present in the solution, some of it will be ionized in accordance with the equation

$$\mathrm{H\,A} \rightleftarrows \overset{+}{\mathrm{H}} + \overset{-}{\mathrm{A}} \qquad (9)$$

Therefore

Activity of undissociated amino acid = (concentration of amino acid −

        concentration of $\overset{+}{\mathrm{Na}}$ − concentration of $\overset{+}{\mathrm{H}}$*) γ_u (10)

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* Since in electrometric measurements, hydrogen ion activity rather than concentration is measured, the activity must be divided by the activity coefficient in order to obtain the numerical value for hydrogen ion concentration. Since hydrogen ion activity coefficients at 0° are not available, those for 25° were used. However, numerically no great error is introduced by assuming the hydrogen ion concentration as equal to the activity.

Similarly for the dissociated amino acid:

ty of dissociated amino acid = (concentration of $\overset{+}{Na}$ + concentration of $\overset{+}{H}$) γ_-

where $\gamma_- =$ the activity coefficient of the aspartate or of the glutamate ion.

Substituting data obtained by Schmidt, Kirk, and Appleman (14) in the determination of the dissociation constants of glutamic acid and of aspartic acid at $0°$ we have for glutamic acid:

$$pK_{a_1} = 3.691 + \log \frac{(0.04 - 0.0094 - 0.0002)\, 0.615}{(0.0094 + 0.0002)\, 0.725} = 4.12 \qquad (12)$$

$$pK_{a_1} = 3.957 + \log \frac{(0.04 - 0.0149 - 0.0001)\, 0.632}{(0.0149 + 0.0001)\, 0.673} = 4.15 \qquad (13)$$

$$pK_{a_1} = 4.164 + \log \frac{(0.04 - 0.0184 - 0.00006)\, 0.644}{(0.0184 + 0.00006)\, 0.648} = 4.21 \qquad (14)$$

The average value for pK_{a_1} obtained from the above calculations is 4.16. Schmidt, Kirk, and Appleman (14) report $pK'_a = 4.14$ for glutamic acid at $0°$. The difference between the value for pK'_a and pK_a is less than the experimental error in determining pK'_a.

The numerical values for γ_u used in the above calculation were obtained by interpolating from a curve obtained by plotting the values for γ_u found by Hoskins, Randall, and Schmidt (9) against the corresponding molar concentrations. The following procedure was employed in calculating γ_-. Values for γ, the activity coefficient of monosodium glutamate, were interpolated from a curve obtained by plotting data given by Hoskins, Randall, and Schmidt (9). The activity coefficients of the sodium ion were interpolated from a curve obtained by plotting the activity coefficients against the molar concentrations (since we are dealing with a uni-univalent salt, the ionic strength is numerically equal to the molar concentration) which are given by Lewis and Randall ((8) p. 382). Their values are for $25°$. A slight error is introduced by using these data for $0°$ calculations. Substituting the above values for $\gamma_{Na\text{-glut.}}$ and $\gamma_{\overset{+}{Na}}$ in the equation ((8) p. 329)

$$\gamma = (\gamma_+^{\nu_+}\, \gamma_-^{\nu_-})^{\frac{1}{\nu}} \qquad (15)$$

values for γ_- are obtained which are then plotted against the corresponding molar concentrations and the desired values for γ_- interpolated.

Similar calculations for aspartic acid yield the following results.

$$\mathrm{pK}_{a_1} = 3.596 + \log \frac{(0.04 - 0.0148 - 0.0002)\,0.498}{(0.0148 + 0.0002)\,0.700} = 3.67 \quad (16)$$

$$\mathrm{pK}_{a_1} = 3.824 + \log \frac{(0.04 - 0.0185 - 0.00015)\,0.516}{(0.0185 + 0.00015)\,0.68} = 3.76 \quad (17)$$

$$\mathrm{pK}_{a_1} = 4.0154 + \log \frac{(0.04 - 0.0228 - 0.0001)\,0.559}{(0.0228)\,0.658} = 3.82 \quad (18)$$

The average value for pK_{a_1} is 3.75. Schmidt, Kirk, and Appleman (14) report $\mathrm{pK}'_a = 3.77$. The difference between the values for pK_a and pK'_a is less than the experimental error in determining pK'_a.

The results indicate that in so far as the primary acid dissociation constants of aspartic acid and of glutamic acids are concerned, no appreciable error is introduced when the apparent dissociation constant is used instead of the true dissociation constant. This is equivalent to stating that in the calculation of this dissociation constant concentration may be employed instead of activity.

At the suggestion of one of us, Dr. W. M. Hoskins has kindly calculated the values for the primary acid dissociation constant of aspartic acid and of glutamic acid on the basis of the freezing point measurements which were reported by Hoskins, Randall, and Schmidt (9). Tables III and IV of their paper give values of m, m_i, m_u, and γ_u. From Table II of a paper by Randall and Young (17) corresponding values of m_i and $m_i^2\gamma_i^2$ were calculated by him in a manner similar to that described by Randall and Allen (18) and by Hoskins, Randall, and Schmidt (9). Values of m at 0.01, 0.02, and 0.03 M were arbitrarily chosen and the necessary interpolations were made in order to obtain the corresponding values of $m_i^2\gamma_i^2$, m_u, and γ_u. These values together with those of $K_a = \dfrac{m_i^2\gamma_i^2}{m_u\gamma_u}$ are shown in Table II.

The values of K_a are dependent upon the value of the dissociation constant originally assumed in calculating the theoretical freezing

point curves from which, together with the experimental freezing point lowering, the quantities above are derived (see pp. 226–227 of Hoskins, Randall, and Schmidt's article (9)). The calculations in Table II are based on $K_a = 2 \times 10^{-4}$ for aspartic acid and $K_a = 6 \times 10^{-5}$ for glutamic acid.[3] For limited variations on either side of these values the ratio $\dfrac{K_a \text{ (calculated)}}{K_a \text{ (chosen)}}$ is equal to 1.9 for aspartic acid and 1.5 for glutamic acid.

Similarly the values of pK_a calculated earlier in this paper from a combination of electrometric and freezing point data depend on the value of K_a chosen in calculating γ_u. If 1.5×10^{-4} be chosen as the dissociation constant of aspartic acid, the average value of pK_a (calculated) is 3.80 instead of 3.75 when 2×10^{-4} is

TABLE II

Calculations of Dissociation Constants from Freezing Point Data

	m	m_u	m_i	γ_u	$m_i^2 \gamma_i^2$	K_{a_1}	pK_{a_1}
Aspartic acid	0.01	0.00860	0.001370	0.620	1.75×10^{-6}	3.28×10^{-4}	3.48
	0.02	0.01800	0.001980	0.528	3.60×10^{-6}	3.78×10^{-4}	3.42
	0.03	0.02753	0.002473	0.481	5.52×10^{-6}	4.17×10^{-4}	3.38
Glutamic acid	0.01	0.00923	0.000767	0.701	0.53×10^{-6}	8.20×10^{-5}	4.09
	0.02	0.01992	0.001100	0.645	1.13×10^{-6}	8.79×10^{-5}	4.06
	0.03	0.02867	0.001325	0.617	1.65×10^{-6}	9.32×10^{-5}	4.03

used. If 2×10^{-5} be chosen as the dissociation constant of glutamic acid, the average value of pK_a (calculated) is 4.20 instead of 4.15 when 6×10^{-5} is used.

In the calculations shown by equations (16) to (18) of this paper the values of γ_u were derived by arbitrarily assuming a value of K_a for aspartic acid by Hoskins, Randall, and Schmidt (9). That this assumption does not lead to an appreciable error in our calculations is seen from the fact that a slight change in the chosen value of K_{a_1} does not materially influence the value of pK_{a_1} calculated since any small variation in the value of γ_u does not materially influence the numerical result of the logarithmic part of these equations. The same reasoning applies also to glutamic acid.

[3] See foot-note to pp. 227 and 228 of Hoskins, Randall, and Schmidt's article (9) for reason as to why these constants were used.

The pK_{a_1} values of aspartic acid and of glutamic acid calculated from freezing point data are probably not as accurate as those which were presented earlier in the paper.   This is probably due in part to the assumptions made by Hoskins, Randall, and Schmidt (9) that aspartic acid and glutamic acid may, with respect to the primary acid dissociation constants, be regarded as simple weak acids rather than as amphoteric electrolytes.   However, no other method for treating freezing point data appears at the present time available.

Unless otherwise stated, the notations used in this paper have the same meaning as those given in Lewis and Randall (8).

SUMMARY

1. The apparent dissociation constants for dl-phenylalanine and dl-3,4-dihydroxyphenylalanine have been determined.

2. Values for $(\Delta F°)'$, the apparent free energy change when the amino acid is in the standard state, corresponding to K'_a and K'_b of such of the naturally occurring amino acids for which the apparent dissociation constants are available, have been calculated.

3. Values for $\Delta S'$, the apparent change in entropy of the system due to ionization, have been calculated for alanine, glycine, arginine, histidine, lysine, aspartic and glutamic acids, and diiodotyrosine.

4. Calculations have been carried out which show that no appreciable error results when concentration is used instead of activity in calculating the primary dissociation constant of aspartic acid and of glutamic acid.

5. Comparisons have been made between the values of K_a of aspartic acid and of glutamic acid calculated from freezing point data and those calculated from a combination of electrometric and freezing point data.

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A SOURCE OF ERROR IN THE DETERMINATION OF AMIDE NITROGEN IN PLANT EXTRACTS*

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No direct method has yet been devised that permits more than a rough estimate of the asparagine content of plant extracts, although the common occurrence of asparagine and its importance in the nitrogenous metabolism of plants (1) make it highly desirable to have a reliable method for this determination. Because of the lack of a direct method recourse has usually been had to an indirect method founded upon the observations of Sachsse (2), in which a sample of the extract is subjected to the hydrolytic action of boiling 4 per cent hydrochloric acid for 4 hours.[1] The ammonia that is produced by this treatment is taken as a measure of the nitrogen in the form of amides. This is not, however, a measure of the asparagine content since other amides, in particular glutamine, may occur in plants; furthermore, other substances yield ammonia under the conditions described and one of them, nitric acid, which is commonly present in plants, in certain cases may introduce considerable uncertainty into the results.

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* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.

The following paper by Chibnall and Miller describes results closely resembling our own. We are indebted to Dr. Chibnall for communicating them to us and for consenting to simultaneous publication.

[1] There is little agreement in the literature regarding the exact conditions of this hydrolysis. Sachsse used approximately 3 per cent hydrochloric acid and boiled the material for either 1 or 3 hours. Chibnall used 4 per cent hydrochloric acid and boiled the material for 4 hours (1). See also Vickery (3). The Committee on Methods of Chemical Analysis of the American Society of Plant Physiologists recommend boiling with 6 per cent hydrochloric acid for 2.5 hours (4).

The observation that nitric acid may interfere with the determination of amide nitrogen was made in the course of an examination of the amide content of an extract from 50 kilos of fresh tobacco leaf of unusually high nitrate content.[2]   It was shown some years ago by one of us that the so called amide nitrogen of the protein gliadin was completely liberated by hydrolysis for 24 hours with 0.2 N hydrochloric acid (5).   Stronger acid concentrations increased the velocity of the amide hydrolysis but gave rise to only minimal increases in the proportion found, and these increases were ascribed to the secondary decomposition of amino acids (see in this connection Vickery and Osborne (6) p. 414).   When the

TABLE I

Effect of Different Concentrations of Hydrochloric Acid on the Total Ammonia Produced in 8 Liters of Extract Prepared from 50 Kilos of Fresh Tobacco Leaf

Acid concentration	Total ammonia N after hydrolysis	
	4 hrs. hydrolysis	20 hrs. hydrolysis
N	*gm.*	*gm.*
0.2		4.03
1.0	4.53	5.39
1.5	4.44	5.48
2.0	5.52	6.44
2.5	5.52	9.62
3.0	7.96	9.58
3.5		10.76
4.0		11.91
5.0		13.21

tobacco extract mentioned above was hydrolyzed for 20 hours by hydrochloric acid, widely different results were obtained according to the concentration of the acid chosen.   The data in Table I show that over 3 times as much ammonia nitrogen was liberated

[2] This extract and those from partially and fully cured tobacco mentioned later were prepared in the course of a chemical study of the curing process, the preliminary results of which are in preparation for publication.   The 50 kilos of fresh leaf contained 288 gm. of nitrogen, of which 105 gm. or 35.2 per cent were soluble in hot water.   Of the water-soluble nitrogen, 17.4 per cent was nicotine nitrogen, 40.2 per cent was nitrate nitrogen, 2.2 per cent was ammonia nitrogen, 1.9 per cent was amide nitrogen, and 11.3 per cent was amino nitrogen.

in 20 hours by 5 N acid as by 0.2 N, while nearly twice as much ammonia nitrogen was liberated in 4 hours by 3 N acid as was set free by 1 N acid in the same period. Because of the high proportion of nitrate nitrogen in this extract we investigated the possibility that nitric acid might be responsible for these anomalous results. As an index of the presence of the substance that interferes with the determination of the true amide nitrogen the increased amount of ammonia produced by severe hydrolysis over that produced by mild hydrolysis was adopted. Hydrolysis by 0.2 N hydrochloric acid for 20 hours or by 1 N hydrochloric acid for 4 hours was regarded as mild; hydrolysis by 5 N acid for 20 hours was regarded as severe.

TABLE II

Effect of Presence or Absence of Nitrate on the Total Ammonia Produced in 8 Liters of Fresh Tobacco Leaf Extract by Hydrolysis with 0.2 N and 5.0 N Hydrochloric Acid

Total ammonia N, nitrate present		Total ammonia N, nitrate absent	
Hydrolyzed by 0.2 N HCl	Hydrolyzed by 5.0 N HCl	Hydrolyzed by 0.2 N HCl	Hydrolyzed by 5.0 N HCl
gm.	*gm.*	*gm.*	*gm.*
4.46	13.21	4.35	4.35
4.44	13.90	4.42	4.40
		4.35	5.51*

* The nitrate was incompletely removed from this sample owing to the use of insufficient nitron.

Samples of the tobacco leaf extract were treated with nitron acetate according to the standard method (7) for the precipitation of nitron nitrate. The precipitate was in each case removed and the filtrate was made faintly alkaline to phenolphthalein by sodium hydroxide. The bulk of the excess nitron was then centrifuged off and the remainder was extracted by ether. The filtrate was faintly acidified to Congo red and aliquot parts of the nitrate-free samples were subjected to mild and to severe hydrolysis. The data are given in Table II and show that the substance responsible for the production of extra ammonia nitrogen had been entirely removed by the nitron.

Additional evidence that the interfering substance was nitric

acid was secured by the examination of a somewhat unusual specimen of cured tobacco that contained no nitrate. A hot water extract of this material gave a negative diphenylamine reaction. On mild hydrolysis it yielded 0.45 per cent of total ammonia nitrogen and on severe hydrolysis 0.38 per cent. Samples of this extract were subjected to severe hydrolysis in the presence of potassium nitrate. Under these conditions an increase in the production of ammonia was expected; on the contrary, however, the total ammonia nitrogen produced was depressed to 0.28 per cent. This unlooked for result suggested that the phenomenon encountered in fresh leaf extract was more complex than it had at first appeared and it was therefore subjected to closer study.

Ammonia is not produced in significant amounts when potassium nitrate is boiled with 5 N hydrochloric acid. When a sample of potassium nitrate that contained 5 mg. of nitrogen was boiled for 20 hours with 20 cc. of 5 N hydrochloric acid, less than 0.05 mg. of ammonia nitrogen was produced. Furthermore when 0.5 mg. of nitrogen as ammonium chloride or sulfate was added and the mixture boiled for 20 hours, less than 0.05 mg. of ammonia could then be detected. Ammonia is therefore oxidized under these conditions by the mixture of hydrochloric and nitric acids and this observation accounts for the loss in total ammonia observed when nitrate was added to the extract of nitrate-free cured tobacco and the mixture subsequently hydrolyzed.

The behavior of the extract of cured tobacco was, however, markedly different from that of the fresh leaf extract above mentioned. A sample of the latter extract was freed from nitrate by means of nitron as already described and divided into three equal parts. Part 1 was boiled for 5 hours with 1 N hydrochloric acid, Part 2 was boiled for 20 hours with 5 N hydrochloric acid. To Part 3 excess of potassium nitrate was added and it was then boiled 20 hours with 5 N hydrochloric acid. Parts 1 and 2 each gave the equivalent of 4.40 gm. of ammonia nitrogen in 8 liters of extract, *i.e.* results practically identical with the experiments reported in Table II, while Part 3 gave 13.9 gm. of ammonia nitrogen in 8 liters of extract, a result similar to the last reported in Table I. The anomalous production of ammonia by hydrolysis with hydrochloric acid in the presence of nitrate appears therefore to be due to some substance or substances in fresh leaf extracts which are not present in cured tobacco.

In order to investigate the effect of curing on this hypothetical substance extracts were made from four lots of tobacco leaf at each of three definite stages of curing and also after fermentation.[3] Each lot was derived from 50 kilos of identical fresh leaf material and the extracts are strictly comparable *inter se* and with the extract of fresh leaf mentioned above. The data on the ammonia produced by hydrolysis by 1 N hydrochloric acid for 4 hours and by 5 N hydrochloric acid for 20 hours are given in Table III together with data for the preformed ammonia nitrogen. There is a marked and rapid production of amide nitrogen when the leaf is hung in the curing sheds for about 12 days or until it has become yellow.

TABLE III

Effect of Curing on the Substance in Green Tobacco Leaf that Promotes the Reduction of Nitrate to Ammonia

Each lot was the equivalent of 50 kilos of fresh leaf and the extract was made to 8 liters.

Stage of curing	Ammonia N before hydrolysis	Total ammonia N after hydrolysis	
		Hydrolyzed 4 hrs. by 1 N HCl	Hydrolyzed 20 hrs. by 5 N HCl
	gm.	*gm.*	*gm.*
Fresh leaf................................	2.19	4.0	13.6
Yellow stage.............................	21.9	34.7	35.3
Brown "	21.0	40.2	39.2
Fully cured..............................	21.6	40.7	37.6
Fermented...............................	55.3	61.1	45.1

This is to be expected and the process continues subsequently at a lower rate. But the striking point is that practically no "extra ammonia" is produced by severe hydrolysis after the fresh leaf has passed through the yellow stage. On the contrary there is a diminution in the ammonia liberated when conditions of severe hydrolysis are employed and this diminution we attribute to direct

[3] Tobacco of the type described here is grown in Connecticut under shade tents. The leaves are picked as they mature, are strung on cord, and suspended on racks in large sheds for many weeks. The leaves first become yellow, then brown, and finally reach a stage recognized as fully cured. They are then packed carefully into large piles where the final process of fermentation occurs.

oxidation of ammonia in the presence of hydrochloric and nitric acids. The point emphasized here is that the substance in fresh leaf which, in the presence of nitrate, seems to be responsible for the production of extra ammonia during severe hydrolysis with hydrochloric acid, largely disappears during the very early stages of the curing process.

In order to discover what share the hydrochloric acid has in the production of this extra ammonia, sulfuric acid was substituted as a hydrolyzing agent. In Table IV are given data which show that 5 N sulfuric acid produces no appreciable increase in the total ammonia nitrogen of fresh leaf extract over the quantities indi-

TABLE IV

Effect of Different Concentrations of Hydrochloric and of Sulfuric Acid on the Production of Ammonia

	Hydrolyzed 4 hrs. by 1 N HCl	Hydrolyzed 20 hrs. by 5 N HCl	Hydrolyzed 6 hrs. by 2 N H₂SO₄	Hydrolyzed 20 hrs. by 5 N H₂SO₄
	gm.	*gm.*	*gm.*	*gm.*
Ammonia N produced in extract from 50 kilos fresh tobacco leaf	4.53	13.21	4.61	4.34
	4.44	13.91	4.45	4.66
	4.36	13.61	4.38	4.40
				4.52
Ammonia N produced in extract from 100 gm. nitrate-free cured tobacco	0.46	0.37	0.41	0.41
	0.45	0.39	0.47	0.46
	0.45	0.39	0.43	0.49
				0.47

cated by 2 N sulfuric acid or by 1 N hydrochloric acid, even when the hydrolysis is prolonged to 20 hours. Furthermore, when an extract of nitrate-free cured tobacco is hydrolyzed for 20 hours with 5 N sulfuric acid, there is no diminution in the ammonia nitrogen although slight unaccountable losses of ammonia occur if 5 N hydrochloric acid is used. When a small quantity of potassium nitrate was boiled for 20 hours with 5 N sulfuric acid no ammonia whatever was produced. It seems clear therefore that the anomalous ammonia originates from the nitrate in fresh tobacco leaf extract under the combined influences of hydrochloric acid and another easily oxidized constituent of the extract.

No evidence has been secured of the identity of this easily oxidized constituent.   It is not glucose.   The curious behavior of fresh leaf extracts can be simulated experimentally since mixtures of 5 N hydrochloric acid with potassium nitrate and capryl alcohol produce appreciable amounts of ammonia when boiled. Also if capryl alcohol and potassium nitrate are added to the nitrate-free extract of cured tobacco mentioned above and this is hydrolyzed by 5 N hydrochloric acid, 2 to 3 times as much ammonia is produced as is formed when the capryl alcohol is omitted. If 5 N sulfuric acid is employed instead there is no extra ammo-

TABLE V

Effect of Capryl Alcohol on Production of Ammonia from Nitrate

	NO_3-N added	Capryl alcohol added	Hydrolyzed 20 hrs. by 5 N HCl	Hydrolyzed 20 hrs. by 5 N H_2SO_4
	mg.	*drops*	*gm.*	*gm.*
Ammonia N produced in extract equivalent to 100 gm. nitrate-free cured tobacco. Samples equivalent to 0.2 gm. were used	3	0	0.20	0.46
	3	0	0.30	0.45
	3	0	0.33	
	3	10	0.89	0.45
	3	10	0.80	
	3	5	0.65	
			mg.	
Ammonia N produced from potassium nitrate solution	3	0	0.00	
	3	10	0.56	
	3	10	0.56	
	3	5	0.43	

nia formed in the presence of capryl alcohol, and a comparison of the data (see Table V) for sulfuric acid with those for hydrochloric acid indicates that, in the absence of capryl alcohol, oxidation of ammonia occurs.

It appears, from the above considerations, that hydrochloric acid is an unsuitable reagent for the hydrolysis of amides in plant extracts and may lead to uncertain results if nitrates are likewise present.   An inspection of our data suggests, however, that the so called Sachsse method, in which approximately normal hydrochloric acid is used and hydrolysis is continued for 4 hours, actually gives results not far from the truth.   Moreover we have found that

asparagine added to fresh tobacco leaf extract can be recovered to the extent of 92 ± 5 per cent when hydrolysis is conducted according to this method (8). But the data also suggest that under these conditions there is a compensation of errors. The direct oxidation of ammonia by the mixture of nitric and hydrochloric acids is compensated by the extra ammonia formed by reduction of nitric acid under the influence of the easily oxidized unknown substance. Satisfactory recoveries of added asparagine may therefore be obtained if the conditions of hydrolysis are rigidly adhered to. It seems more conservative, however, to avoid the necessity of dependence upon such an adventitious compensation by employing sulfuric acid exclusively for the hydrolysis during determinations of amide nitrogen in plant extracts. We therefore suggest that 2 N sulfuric acid be substituted for the approximately N hydrochloric acid of Sachsse and that the time of hydrolysis be increased to 6 hours. Under these conditions accurate and reproducible results are easily secured and no interference from nitrate is to be apprehended. There is nothing novel in this suggestion as sulfuric acid has frequently been used for this purpose (9) and considerable latitude is permissible both in acid concentration and in time of hydrolysis. Similar latitude is not permissible, however, if hydrochloric acid is employed.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Preparation of Extracts*</div>

The fresh tobacco leaves were dropped into boiling water in a large vessel, until a convenient quantity had been added, at such a rate that boiling was not interrupted. Sufficient dilute sulfuric acid was added to the water so that the reaction of the fluid was approximately pH 4.0. This was done so as to avoid loss of nicotine during boiling and the subsequent concentration (10). After boiling for $\frac{1}{2}$ hour the extracted leaves were removed, drained, enveloped in cloth, and pressed on the hydraulic press. Meanwhile more leaves were added to the same liquor and the process repeated until the entire quantity of 50 kilos had been extracted. In this way the total volume of extract was kept within convenient limits. The press-cakes were shredded into boiling distilled water, boiled for a few minutes, and again pressed. This process was repeated once more. The three extracts were collected quanti-

tatively, combined, passed through a filter, and concentrated *in vacuo*. Tests showed no nicotine in the distillate. The extract was again filtered, and made to 8 liters. It was preserved with toluene.

Extracts of the partially and fully cured leaves were prepared under identical conditions. The sample of nitrate-free cured tobacco was extracted in a similar way but, as only 5 gm. were available, the suspension in boiling water was centrifuged and the material was reextracted repeatedly with boiling water. The extract was made to 250 cc. and 10 cc. samples were used for the experiments. In all cases the selection of aliquots was made so as to yield amounts of ammonia within the limits of accurate colorimetric estimation.

Determination of Total Ammonia Nitrogen

10 cc. of the concentrated extract were diluted to 200 cc. and of this solution either 5 cc. or 10 cc. were used for the hydrolysis experiments. In order to conduct the hydrolysis with 5 N acid, 10 cc. of 10 N acid, 5 cc. of extract, and 5 cc. of water, or 10 cc. of 10 N acid and 10 cc. of extract, were taken, and the mixture was boiled under a reflux condenser for the required period. It was then transferred to a 300 cc. Kjeldahl flask and the ammonia present was determined as described by Vickery and Pucher (8). When the hydrolysis was conducted with lower concentrations of acid a proportionally smaller amount of 10 N acid was used and the balance of 10 cc. was made up with water. In those experiments in which nitrate was added 3 cc. of a standard solution of potassium nitrate containing 1 mg. of nitrogen per cc. were substituted for 3 cc. of the water. This provided approximately a 30-fold excess of nitrate nitrogen over the true amide nitrogen of the extract and closely imitated the conditions in the fresh leaf extract.

SUMMARY

Although the determination of the amide nitrogen of plant extracts has always been carried out by an estimation of the ammonia produced by mild hydrolysis, there is little agreement in the literature as to the exact conditions under which this hydrolysis should be conducted. Evidence is presented that widely varying results are secured for the total ammonia nitrogen of fresh tobacco leaf

extracts after hydrolysis, according as the concentration of the hydrolyzing acid is chosen. This variability has been shown to be due to the use of hydrochloric acid in the presence of the nitrate that is a normal constituent of tobacco leaf extract. Fresh tobacco leaf appears to contain an easily oxidized substance which promotes the reduction of nitric acid to ammonia in the presence of excess of hydrochloric acid. This substance largely disappears during the curing of the tobacco leaf for commercial use. No evidence has been secured of the identity of this unknown substance but its behavior can be simulated by capryl alcohol.

A second factor that leads to variability in the determination of amide nitrogen consists in the direct oxidation of ammonia by the mixture of hydrochloric and nitric acids. It so happens that, under the conditions of hydrolysis generally known as the Sachsse method; these two factors approximately compensate each other and asparagine added to a nitrate-containing plant extract can be satisfactorily recovered, provided that definite conditions of hydrolysis are rigidly maintained. The results of this paper do not, therefore, necessarily cast doubt upon the accuracy of published data for the amide content of plant tissues that likewise contain nitrates. It is suggested, however, that the use of hydrochloric acid for amide hydrolysis under these conditions should be abandoned and that sulfuric acid be substituted. Data are presented which show that considerable latitude both in the concentration of the acid and the time of hydrolysis is then permissible.

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SOME OBSERVATIONS ON THE DISTRIBUTION OF NITROGEN IN PLANT EXTRACTS THAT CONTAIN A HIGH PROPORTION OF NITRATE NITROGEN*

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In this paper we wish to call attention to certain abnormalities in the distribution of nitrogen in the aqueous extract of a particular sample of perennial rye-grass (*Lolium perenne*) which we have obtained during the course of an extended investigation into the nitrogenous constituents of forage grasses. These abnormalities were eventually traced to the presence of unusually large amounts of nitrate nitrogen in the extract, and have led us to revise in certain details the methods by means of which we partition the nitrogen into the now commonly accepted groupings.

The grass in question was obtained from a sewage farm, and was cut 1 hour after sunrise. Although growth was luxuriant, the grass was known to be of very poor agricultural quality. At the time of collection it stood some 12 inches high, and, to insure that the sample contained only leaf blades and no stems, the scythe used for cutting was held some 6 inches above the ground. The initial distribution of nitrogen in the aqueous extract, as given in

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* During the course of the work described in this paper, we communicated with Dr. Vickery, of the Connecticut Agricultural Experiment Station, our discovery of abnormally high nitrate concentration in grass, and its effect on the values obtained for certain other nitrogenous groupings. He informed us that he was himself encountering similar difficulties with tobacco extracts that contained high nitrate nitrogen, and suggested an exchange of experiences and simultaneous publication of results. We feel particularly indebted to Dr. Vickery for advanced proofs of papers on the determination of total nitrogen of plant extracts in the presence of nitrates (1) and on the determination of nitrate nitrogen in tobacco (2).

the first column of Table I, was obtained by methods which for the purposes of this paper need only the following brief description.

Ammonia N—This was obtained by distillation with magnesia in the usual Kjeldahl nitrogen apparatus.

Amide N—This represents the increase of ammonia N after hydrolysis with 4 per cent HCl for 2 hours.

Other Amide N—This represents the further increase of ammonia N after hydrolysis with 20 per cent HCl for 16 hours.

Amino N—The amino N was estimated by Van Slyke's method.

TABLE I

Distribution of Nitrogen in Aqueous Extracts of Normal and Abnormal Samples of Perennial Rye-Grass

The figures given are in percentages of total leaf nitrogen.

	Abnormal grass (from sewage farm)	Normal grass (dressed with 112 lb. Ca-$(NO_3)_2$ per acre)
Total water-soluble N	26.20	20.00
Ammonia N	1.21	0.62
Amide N	1.08	1.20
Other amide N	3.48	0.58
Amino N	5.82	5.02
Peptide N	0.47	5.26
Nitrate "	{ 4.11 / 12.70*	0.00

*The final value was obtained by the method of Vickery and Pucher (2).

Peptide N—Peptide N represents the increase of amino N after hydrolysis with 20 per cent HCl.

Nitrate N—Nitrate N was estimated as ammonia after reduction by Devarda alloy in alkaline solution at room temperature. The procedure was essentially that of Chibnall (3).

It was noted at the time that the nitrate determinations were very irregular, but our attention became focussed on the extravagant figure for the other amide N, which suggested the presence of relatively large amounts of substances having a composition very different from that usually encountered in leaf extracts. Knowing the origin of the grass we at first suspected the presence of urea, which might have been absorbed by the root system of the grass,

and stored unchanged in the blades. A search for urea by the soy bean urease method showed, however, that this substance was absent.

The extract was then fractionated by Vickery's (4) method, which can be described briefly as follows (see also Table II): Baryta was added in excess to the extract and the precipitate was filtered off. The precipitate, which consisted chiefly of non-nitrogenous acids, was not analyzed further. To the barium-free filtrate (Fraction 1) were then added mercuric acetate and sodium carbonate in slight excess, followed by the addition of 2 volumes of alcohol. The precipitate, which contained the mercury carbamates of all those substances that have a free α-amino group,

TABLE II.

Showing Fractionation of High Nitrate Samples of Grass by Vickery's Method

The figures given are in gm. of nitrogen.

	Original extract	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
Total N..........................	37.5	34.5	15.9	5.4	7.6	14.2
Nitrate N*.....................	18.2	18.2	2.9	0.0	0.8	12.8
Ammonia ".....................	1.9	1.4	1.4	1.0	0.4	0.0
Amide N...:...................	1.3	0.9	0.8		0.6	0.3
Other amide N.................	5.0	3.6	1.3	0.0	0.6	0.4
Amino N........................	8.4	7.8	6.8	1.0	4.8	0.2
Peptide N......................	0.6			1.0		

* Obtained by the method of Vickery and Pucher (2).

was decomposed with hydrogen sulfide, and the resulting solution (Fraction 2) was further fractionated by phosphotungstic acid into basic substances (Fraction 3) and monoamino acids (Fraction 4). The filtrate from the mercury precipitation was freed from the metal with hydrogen sulfide, acidified with acetic acid, concentrated, and treated with phosphotungstic acid (in the presence of 5 per cent H_2SO_4) to remove basic substances that have no free amino group. This fraction was very small and was not investigated further. The filtrate was freed from phosphotungstic and sulfuric acids (Fraction 5).

To our surprize this fractionation led to the loss of nearly all of the other amide N, and drew our attention to the large amount of

nitrogen in Fraction 5, which should normally be practically nitro-
gen-free.

Nitrate determinations on this fraction by the Devarda alloy
method which we were employing at that time were very irregular,
and led us to suspect that the original grass extract may have
contained far more nitrate nitrogen than we had thought. On
investigation this was found to be the case, and the initial low and
irregular values for the nitrate N were shown to be due to the
employment of insufficient Devarda alloy and of too low a tempera-
ture during the reduction. By increasing the amount of alloy to
1 gm., but still carrying out the reduction at room temperature, a
value for nitrate nitrogen of 8.2 per cent of total leaf N was secured.
It was suspected that this high value was the cause, at any rate in
part, of the abnormal other amide N. The residual liquid from
this nitrate determination was consequently filtered, neutralized
with HCl, again filtered, evaporated to a small bulk, and hydrolyzed
for 16 hours with an equal volume of concentrated HCl. The value
for other amide N fell to 1.97 per cent. Reduction of the nitrate
with 1 gm. of Devarda alloy at 100° raised the nitrate content to
12.1 per cent and the reduced iron method of Vickery and Pucher
(2), which had at this time been communicated to us, even higher
—to 12.7 per cent or 48.5 per cent of the water-soluble N. The
value for other amide N of 1.97 per cent quoted above had there-
fore been obtained in the presence of 4.5 per cent unreduced nitrate,
and there seemed to us, at the time, no doubt that the abnormally
high other amide N was explained by reduction of nitrate during the
HCl hydrolysis. No further experiments were therefore made.

At a later date, when the results given in the preceding paper of
Vickery and Pucher (5) were sent to us in manuscript, we should
have liked to have determined the value for other amide N after
hydrolysis with H_2SO_4 but unfortunately none of the original ex-
tract was available. The only other grass extract then available
which contained as much as 1 per cent of nitrate N was one from a
sample of cocksfoot (*Dactylis glomerata*) which had received 3
times the usual dressing of calcium nitrate (336 pounds per acre).
This contained 0.93 per cent nitrate N and gave the analysis shown
in Table III. Other amide N after hydrolysis with 20 per cent
HCl was 1.43 per cent, whereas after hydrolysis with 5 N H_2SO_4
the value was only 0.48 per cent. This shows that the deter-

mination of other amide N by HCl hydrolysis is quite inadmissible when nitrates are also present.   Furthermore, as the nitrate presumably passes through the nitrite stage during reduction, the determinations of the peptide N after hydrolysis with 20 per cent HCl may be low.

In the preceding paper Vickery and Pucher (5) call attention to the fact that, in the case of tobacco leaf extracts, it is the presence of some easily oxidizable substance which promotes the reduction of the nitric acid, and that this substance, although present in some extracts, is entirely lacking in others.   They have found by experiment that the substance is not glucose; our results not only

TABLE III

Showing Different Values for Other Amide N Obtained by Hydrolysis with 20 Per Cent HCl and 5 N H₂SO₄

Measurements are in percentages of total leaf N.

Total N	13.50
Ammonia N	0.79
Amide N	0.51
Other amide N	
1. Hydrolysis with 20 per cent HCl	1.43
2. " " 5N H₂SO₄	0.48
Amino N	3.?8
Peptide N	1.96
Nitrate "	0.93

confirm this observation, but suggest that the substance, or substances, are probably nitrogenous.

Reverting to the fractionation illustrated in Table II, the first point to be noted is that Fraction 1, which contains all the nitrate N, yields a large proportion of the other amide N.   When this is fractionated by mercuric acetate and sodium carbonate the ultimate filtrate (Fraction 5), which contains the major part of the nitrate N, yields in proportion but very little other amide N.   It is clear therefore that the oxidizable substance does not belong to the groups that would be found in this fraction; *e.g.*, sugars, polysaccharides, and organic substances that possess neither acidic nor basic properties.   It is true that the mercury precipitate (Fraction 2) contains only 1.3 gm. of other amide N, and that there appears to be a loss of some 2 gm. in the fractionation, but if this nitrogen

has its origin in the nitrate N this is to be expected, for the amount of the latter has been reduced from 18.2 gm. in Fraction 1 to only 2.9 gm. in Fraction 2.

It is quite legitimate to conclude therefore that the oxidizable substance, which is clearly absent from Fraction 5, has passed into Fraction 2.   By a similar process of reasoning it would seem that the oxidizable substance has passed into Fraction 4, and not into Fraction 3.   Fraction 3, it is true, contains no nitrate N, but in an experiment in which nitrate was added as KNO_3 in amount equal to more than 10 times the total N in the extract, no other amide N was found.   Unfortunately a sample of Fraction 4 is not now available for further analysis, so that we cannot bring forward definite proof that Fraction 2 gives an increased value for other amide N when enriched with KNO_3, and that this value is decreased to a nominal amount when H_2SO_4 is used instead of HCl for the hydrolysis.   In spite of this we think that we may tentatively suggest that the low values for other amide N in Fractions 2 and 4 are due to the low concentration of nitrate N present in those fractions, and that there has not necessarily been an appreciable loss of other amide N during the fractionation.   On the admittedly scanty evidence that we are able to put forward, however, we are perhaps on firmer ground when we suggest that, in any case, the oxidizable substance or substances are nitrogenous, and it is this one fact alone which prompts us to enter into any discussion at all as to their chemical nature; for being nitrogenous, their presence or absence in the leaf may be connected with the protein metabolism.

This deduction is of great interest, for it appears to offer a logical explanation of certain puzzling results obtained by one of us some years ago when investigating the protein metabolism of the runner bean leaf (6).   It would be inconvenient here to describe in any great detail the experiments quoted in that paper, but briefly, it may be stated that in certain cases starvation of the bean leaves did not lead to the usual accumulation of asparagine (amide N) but gave rise to large amounts of other amide N (see Table IV). In view of our more recent experiences, which have already been quoted in this paper, we feel that these abnormal results can be explained in the following way.

In Case 2 the nitrate N was estimated by reduction at room

temperature (in alkaline solution) with a small amount of Devarda alloy.   Experience suggests that this estimation was low and that, in the experiments on runner bean leaves (3) carried out in 1921, the nitrate N may have been higher than was then suspected. Unfortunately the values for nitrate N in Cases 1 and 3 were not determined.   In the normal leaves (Case 1) the oxidizable substance of Vickery and Pucher was present only in small amounts and was not greatly increased by starvation.   The value for other amide N was therefore low.   In the abnormal leaves (Cases 2 and

TABLE IV

Showing Increases in Various Forms of Non-Protein N Due to Protein Metabolism in Leaves of Normal and Abnormal Plants

Phaseolus vulgaris var. multiflorus.   (Measurements are in percentages of total leaf N.)

Case No.	Type of leaves	No. of days with petiole in water	Total non-protein N	Nitrate N	Ammonia N	Amide N	Other amide N
1	Normal	0	16.75		0.46	1.06	1.44
		4	31.60		0.45	4.08	2.05
Increase			14.85			3.02	0.61
2	Abnormal	0	22.45	2.73	0.44	0.53	0.73
		4	34.14	3.42	0.44	0.49	5.69
Increase			11.69				4.96
3	Abnormal	0	16.75		0.46	1.06	1.44
		4	28.80		0.49	2.29	3.66
Increase			12.05			1.23	2.22

3) however, the amount of this oxidizable substance increased on starvation, consequently there was a large increase of other amide N.   In both of these abnormal cases the nitrate N was undoubtedly higher than usual, which suggests that, in the presence of large amounts of nitrate N, the protein metabolism of the leaf may be modified and may lead to no appreciable increase of true amide N.

Further experiments are clearly necessary before this latter surmise can be accepted, but the very high concentration of nitrate N that we have found in rye-grass, equal to 12.7 per cent of the total N or, calculated as KNO_3, equal to 4.4 per cent of the dry

weight of the leaf shows that, under the appropriate conditions, leaves are capable of storing relatively enormous amounts of nitric acid. We believe the above figures for the proportion of nitrates in grass to be much greater than any hitherto recorded in the literature of leaf analysis with the exception of data that have been obtained on the tobacco leaf (see for example (2)).

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THE FADING OF TROPEOLIN OO IN THE TITRATION OF ORGANIC ACIDS IN URINE

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In a study of urine acidity in tuberculosis (1) the method of Van Slyke and Palmer (2) was used for the determination of the organic acids. This method is based on the fact that at pH 8 the organic acids in the urine are present as salts and that the addition of nearly a full molecule of HCl for each molecule of the salt of the weak acid is necessary to free the weak acid. This change is practically complete at pH 2.7 and the indicator which has been found to be satisfactory, with few exceptions, is tropeolin OO. Occasionally fading of the indicator occurs, especially near the end-point (2.7). This occurred in the urine of three of the eighteen subjects (later a much larger group was studied) used in the above study. Two of these patients belonged to the critically ill group and one to the group not so ill. It was first observed in the night specimen only of one of the critically ill patients. That the ingestion of drugs was probably responsible for this behavior was given first consideration. However, the only medication this patient was receiving at the time was codeine sulfate and tincture of opium. Investigation revealed that the fading phenomenon was not caused by these medicaments, for it was found that the interfering substance was not always present in the urine of this patient under the same medication, that it was not present at all in the urine of other patients receiving exactly the same kind and amount of medication, and that codeine sulfate and tincture of opium added to the urine did not affect the indicator.

The fading of this indicator in the titration of organic acids in urine has been reported by Palmer (3), and after attempts to remove the interfering substance with aluminum hydroxide,

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charcoal, fullers' earth, copper hydroxide, lead hydroxide, and colloidal iron were unsuccessful, he came to the conclusion that when fading occurs with tropeolin OO another indicator must be used. Bromophenol blue was the one suggested. Palmer also states that the ingestion of drugs was not responsible for this difficulty although he does not specify what drugs were eliminated.

As an alternative, bromophenol blue was tried but found impractical with urine of high pigment (a common finding in pathological states) as the color of the indicator was so modified as to make comparison impossible. In fact, under the most optimum conditions this indicator is not nearly so satisfactory as tropeolin OO because the color change at pH 2.7 is rather sluggish in contrast to the sharp end-point of the latter.

It became imperative to find either a more suitable indicator or a method of removing the interfering substance. It was noticed, an observation mentioned also by Palmer, that if the tube in which fading occurred was allowed to stand, in time it became red, first at the surface layer. Furthermore, if in the titration only enough acid was added to change the pH from 8 to just the acid side of litmus and if the titration tube was allowed to stand under the same conditions as the previous tube, the fading of the indicator was not observed nor was the interfering substance removed. The chemical reaction between the indicator and the interfering substance, if judged by the fading phenomenon, does not take place except well on the acid side. It became evident that this substance affecting tropeolin OO was an easily oxidizable substance, especially in an acid solution of pH 2.7 or less, that in the oxidized form it either did not react with tropeolin OO or was volatile, that the fading or alteration to a yellow shade from the red color of tropeolin OO (pH 2.7) can be attributed to the reduction of this azo dye to the hydrazo compound (this is quite a general behavior of red-colored azo compounds with moderate reducing agents), and that the reappearance of the red color on standing is the result of oxidation of the hydrazo compound to the original azo compound.

From these observations a method was found by which the interfering substance could be removed by bubbling air for $\frac{1}{2}$ hour through the urine to which concentrated hydrochloric acid has been added in the proportion of 0.5 cc. to 100 cc. of urine. This

amount of acid brings the pH of most urine to the neighborhood of 2.7. In some urine with a high acidity, bubbling air through the urine without the addition of hydrochloric acid was sufficient to remove the interfering substance, but in others it did not.

Whether this procedure gave erroneous results for organic acids in urine samples that could be titrated in the usual way was next investigated. Each urine specimen was divided into three parts. One part was treated as required by the Van Slyke-Palmer technique; a second portion was aerated for ½ hour and then

TABLE I

Cc. of 0.2 N HCl (Corrected for Blank) Used for Titration of 25 Cc. of Urine

Van Slyke-Palmer method	Aerated without addition of HCl			Aerated with 0.5 cc. concentrated HCl per 100 cc. urine		
		Difference	Per cent difference		Difference	Per cent difference
4.01	3.97	−0.04	−1.0	3.83	−0.18	−4.4
6.99	7.06	+0.07	+1.0	6.45	−0.54	−7.7
2.99	3.07	+0.08	+2.6	2.61	−0.38	−12.6
2.38	2.32	−0.06	−2.5	2.31	−0.07	−2.9
3.68	3.67	−0.01	−0.2	3.40	−0.28	−7.6
6.97	6.84	−0.13	−1.8			
4.56	4.78	+0.22	+4.8			
2.30	2.19	−0.11	−5.0			
6.25				6.17	−0.08	−1.2
8.33				8.09	−0.24	−2.9
6.16				6.07	−0.09	−1.4
3.40				3.47	+0.07	+2.0
6.67				6.50	−0.17	−2.5
Average......		−0.07 to +0.12	−2.1 to +2.8		−0.22	−4.6

treated as the first; to the third, concentrated hydrochloric acid in the proportion of 0.5 cc. to 100 cc. of urine was added and after aeration for ½ hour it was treated as the first.

After many experiments (Table I gives a few examples) it was found that the urine which had been aerated only, always gave values for organic acids that agreed with the Van Slyke-Palmer procedure within experimental error; that the urines that had been aerated with hydrochloric acid gave values either within the range of error or values always slightly less. The limit of experimental

error is placed at 0.2 cc. of 0.2 N acid, although duplicates often check within 0.1 cc. or less. This amount is allowable since adjustment to two end-points (phenolphthalein and tropeolin OO) is required. This small negative difference could well represent volatile acids, since the organic acids are free at pH 2.7 and the volatile acids would be lost. Air freed from carbon dioxide, after passing through the acidified urine, was made to bubble through carbon dioxide-free alkali, and the latter solution titrated from pH 8 to 2.7 with tropeolin OO. The only volatile acid found was carbonic acid; and this was true with urine from diabetics containing acetone and diacetic acid.

The other factors considered to explain this small difference in titration values were the incomplete removal of the carbonates by the Van Slyke-Palmer technique, the effect of air in an acid solution upon uric acid, creatinine, and creatine, and the salt effect from the added chloride ion.

The carbonates appear to be quite completely removed when present in the usual amounts found in urine by the treatment with calcium hydroxide. Urine that had been so treated with calcium hydroxide was acidified to pH 2.7 and aerated with carbon dioxide-free air which was then bubbled through carbon dioxide-free alkali. The alkaline solution was titrated from pH 8 to 2.7 and found to contain carbonic acid in quantities no larger than 0.25 cc. of 0.2 N acid per 100 cc. of urine. Palmer finds that the simple technique of shaking the urine for 15 minutes with calcium hydroxide does not remove all of the carbonates if the latter are present in amounts exceeding 0.5 per cent calculated as bicarbonate. Uric acid, creatinine, and creatine are not altered by this aeration procedure in acid solution. The salt effect on the indicator due to the increase in chloride ion was found to be negligible. The small difference in organic acid values sometimes found after aeration with hydrochloric acid may be due to the pigments of the urine, as they appear to be altered by the hydrochloric acid, for the color becomes intensified. However, the filtrates after calcium hydroxide treatment are no darker than untreated urine.

The nature of this interfering substance is unknown, but probably is of endogenous origin and from reports to date has been found only in pathological subjects, where it is not a constant but a variable finding. Chemically, it is easily oxidized in acid solution.

SUMMARY

1. The aeration of urine in the presence of hydrochloric acid (*a*) will remove an easily oxidizable substance found to react with tropeolin OO; (*b*) will remove carbonates, a necessity when present in quantities greater than 0.5 per cent; (*c*) may give values on an average of 5 per cent lower than does untreated urine. This difference is not due to loss of volatile acids, to incomplete removal of the carbonates, to changes in the uric acid, creatinine, and creatine, nor to the salt effect upon the indicator, but may be due to pigment content.

2. The urinary substance found to react with tropeolin OO is an easily oxidizable substance and may be an unoxidized sulfur compound arising from the destruction of tissue protein and is probably present in other diseases besides tuberculosis.

3. Upon repeated examinations this substance has been found to occur at intervals in 30 to 40 per cent of the very ill phthisical patients.

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PREPARATION AND PROPERTIES OF CRYSTALLIZED
ALKALI SALTS OF *l*-CYSTINE

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A number of the normal salts of *l*-cystine with bases have been described by Neuberg and Mayer (1). Only the Cu salt was obtained in crystals while the salts with Ag, Hg, Pb, and Cd, as well as with Fe, Cr, Sn, and Zn, were amorphous. All of these salts are characterized by their insolubility in water. No data are available on the isolation of salts of the cystine anion with alkali metals. They form the object of the present report.

The principle used in the preparation of these salts is to dissolve the cystine in an alcoholic alkali solution to which just sufficient water to effect solution has been added, and, after filtering from excess cystine, to precipitate the salt by addition of a suitable indifferent solvent. While various solvents, such as acetone, ether, or large amounts of alcohol caused precipitations, these were either oily or amorphous. Only acetonitrile was found to possess the power of initiating regular crystallization in the salt solutions. While the solutions of the different salts require different amounts of the solvent for complete precipitation, a partial substitution of absolute ether for acetonitrile was found expedient in the case of the most soluble of the salts, the K salt.

Considerable experimentation led to the procedures described in the experimental part, and although the conditions given there are by no means unalterable, close adherence to them will avoid difficulties of crystallization. A method of recrystallization was not found and reprecipitation under the conditions of preparation did not prove satisfactory with respect to improvement of purity or crystal form. However, analysis indicated a fair state of purity of the original preparations.

Comparison of the optical rotations of acid solutions of equal concentrations, of the original cystine on the one hand and the salt on the other, showed an almost unchanged recovery of the *l*-cystine from its alkali salt.

Of the three salts prepared, the Na, K, and Li salts, only the Na salt is obtained with 1 molecule of water of crystallization, which is given off at 78° *in vacuo*.   The K salt, on the other hand, is distinguished by its deliquescent nature.   On exposure to the atmosphere it soon gets sticky and loses its structure.   The two other salts merely absorb a little moisture from the air which, however, can be removed by drying.

As regards the crystalline structure, the Na salt is the least satisfactory one, while the K and the Li salts were obtained in at least two different distinct crystal forms.   It seems, however, improbable that these different crystal forms are related to the different optical modifications of cystine, since their quantitative distribution does not agree with the composition of the cystine used (with a maximum content of 6 per cent inactive cystine).   Rather does it seem that in different concentrations different types of crystal growths prevail.

All three salts are very soluble in water and they are also soluble to some extent in the lower alcohols, the solubility in CH_3OH being considerably larger than that in C_2H_5OH, while the alcohol solubilities rapidly increase in the order Li→Na→K salt.

Determinations of optical rotation showed that the aqueous solutions are practically stable for a number of days, while the methyl alcoholic solutions undergo a rapid decomposition, evidenced also by discoloration of the solution and evolution of NH_3. These stability differences between the aqueous and alcoholic solutions undoubtedly are related to differences in the ionic state of the cystine molecule, differences that are accompanied by radical steric and energy changes (2).   By the investigations of Levene and his associates (3), the existence of definite relations between ionization and optical rotation has been generally established. As regards *l*-cystine, earlier observations (4, 5) have indicated already that the rotation in alkaline solution is less than half as large as that in acid solution, and our data on the aqueous solutions of the alkali salts fully agree with those findings.   The rotation of the alcoholic salt solutions, on the other hand, we find to be higher

than that of the acid cystine solution. This large rotatory difference is paralleled, as mentioned above, by a significant difference of chemical stability. A shifting of the ionic equilibrium from the anionic form toward the non-ionized and hybrid ion forms, due to the weaker basicity of the alkali in the alcoholic solution, might account for both phenomena. A specific solvent influence of the alcohol on the rotation might also play a rôle, although observations of Abderhalden and Wybert (6) on the rotation of cystine derivatives (monochloroacetyl-, dichloroacetyl-, dibromoacetyl-, and diiodoacetyl-cystine) indicate that the solvent effect of alcohol on the rotation of cystine is of a minor order. For, by calculating and expressing the rotations given by the authors as specific rotations of the cystine molecule instead of the individual derivative, only comparatively small differences appear between the rotations of these compounds although they were determined partly in HCl and partly in C_2H_5OH.

Finally the stability of the aqueous salt solutions appears noteworthy in view of the known instability of cystine solutions when boiled with excess alkali (*cf.* (7)).

<div align="center">EXPERIMENTAL</div>

The cystine used in most of this work had a specific rotation of $[\alpha]_D^{29} = -192.1°$, corresponding to a mixture of 94 per cent *l*-cystine and 6 per cent inactive cystine (8).

I. Sodium Salt—To 2.5 gm. of cystine, 43.5 cc. of a 0.46 N NaOH solution in 82 per cent C_2H_5OH are added. The solution of sodium cystinate is filtered through a dry filter from excess cystine; 2 volumes of acetonitrile (Eastman) are added and, after brief shaking, the solution is allowed to stand undisturbed for 1 to 2 hours By this time, the milky solution should have changed to a crystalline mass with supernatant clear liquid. The crystals are filtered, washed with some acetonitrile, and freed from excess solvent in a vacuum desiccator (yield about 75 per cent).

In some cases a yellowish oily phase settles to the bottom soon after the solution has been filtered. In this case addition of a little 82 per cent C_2H_5OH will redissolve the oily layer. It is, however, always advisable to add the acetonitrile as quickly as possible after filtering in order to forestall any possible separation. If, soon after addition of the acetonitrile, crystal aggregations

do not begin to appear on the glass walls, seeding with some crystals previously obtained in a test-tube experiment will initiate the crystallization.

The crystals consist of fine needles that have a silky appearance when dry; while under the microscope they appear mostly as irregular conglomerations of branched needles, they form occasionally
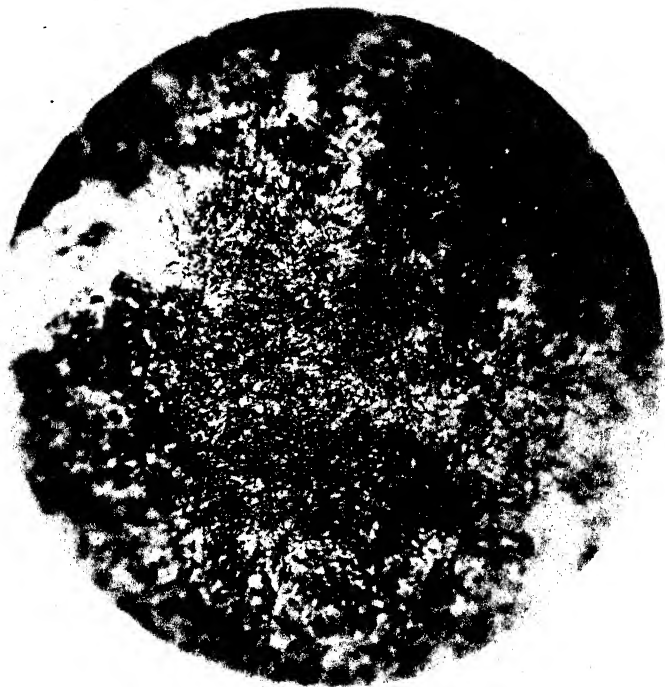

FIG. 1. About $\times$ 80.   Crystals of Na salt

regular radiating groups, visible to the naked eye, on the walls of the glass vessel.   Fig. 1 gives a photomicrograph of the Na salt.

A sample dried to constant weight *in vacuo* over P_2O_5 at 56° gave the following figures.   (All alkali titrations of the salts were carried out with 0.02 N HCl, with methyl red as indicator.)

$C_6H_{10}O_4N_2S_2Na_2$.   Calculated.   Na 16.58
$C_6H_{10}O_4N_2S_2Na_2 + H_2O$.   "   " 15.23
                            Found.   " (volumetric) 15.45, 15.54;
                                        (as Na_2SO_4) 15.12, 15.06

Samples previously dried at 56° to constant weight were now dried to constant weight at 78°, and the loss in weight was assumed to represent H_2O.

$C_6H_{10}O_4N_2S_2Na_2 + H_2O$. Calculated. H_2O 5.96
Found. " 5.90, 6.04

Drying at 100° did not cause any further loss of weight. Above 100° decomposition starts. Samples dried at 78° gave the following analysis.

$C_6H_{10}O_4N_2S_2Na_2$. Calculated. Na 16.18, S 22.54
Found. " (volumetric) 16.48, 16.41
" S (as $BaSO_4$ according to Benedict-
Denis (9)) 21.95, 22.03

These analyses indicate that the Na salt crystallizes with 1 molecule of H_2O.

The salt is very soluble in water. The approximate solubility of the anhydrous salt in anhydrous C_2H_5OH and CH_3OH at 25° was determined by saturating the alcohol with the salt at 30°, cooling to and keeping at 25° for at least 10 minutes with constant shaking, filtering, and titrating the alkali in measured volumes of the filtrate diluted with water.

C_2H_5OH solution. Found. 0.017 gm., 0.014 gm. per 100 cc.
CH_3OH " " 2.40 " 2.28 " " 100 "

The specific rotation of the anhydrous salt was determined and calculated, for comparison, for cystine; 0.2506 gm. of salt dissolved to 25 cc. in H_2O gave $[\alpha]_D^{25} = -89.6°$, $[\alpha]_D^{27} = -90.5°$ (after 24 hours); 0.2500 gm. of salt dissolved to 25 cc. in CH_3OH gave $[\alpha]_D^{25} = -265°$, $[\alpha]_D^{27} = -250°$ (after 24 hours). While after 24 hours the aqueous solution shows no signs of decomposition, the methyl alcoholic solution has turned yellow and NH_3 is being given off.

II. Potassium Salt—To 2.5 gm. of cystine 20 cc. of a 1 N solution of K in absolute CH_3OH are added. The solution of potassium cystinate is filtered from excess cystine. A mixture of 75 cc. of absolute ether and 75 cc. of acetonitrile is added, and, after brief mixing, the whole is left in a refrigerator overnight. The crystalline mass is filtered and brought into a desiccator immedi-

ately as the product is deliquescent in the air.   With special care, the theoretical yield is obtained.

Under the conditions described the crystallization usually begins with the separation of droplets from the milky emulsion that first results on addition of the precipitating mixture.   These droplets gradually assume a crystalline structure as shown in Fig. 2. Another crystal form also appears, consisting of irregular or plantlike conglomerations of spear-shaped growths.   The latter form may be obtained especially well developed by adding at first about 3 volumes of the precipitating reagent to the alkaline cystine solution, resulting in the formation on the glass walls of crystallization nuclei of the first type, by pouring off the supernatant liquid after it has become clear, and adding to this at least twice its volume of precipitant; the crystals of the second type will now form in the more diluted solution.   Fig. 3 shows this second type of the potassium salt crystals.

For analysis the salt was dried to constant weight over P_2O_5 *in vacuo* at 78° (at 56° the same result was obtained).

$C_6H_{10}O_4N_2S_2K_2$.   Calculated.   K 24.59, S 20.16
Found.   " (volumetric) 24.63, 24.64; K (as K_2SO_4) 24.49; S (as $BaSO_4$ after oxidation in Parr Oxygen Bomb) 19.77.

The K salt also is very soluble in water.   The solubilities in C_2H_5OH and CH_3OH were determined in the manner described for the Na salt.

C_2H_5OH solution.   Found.   1.75 gm., 1.91 gm. per 100 cc.
CH_3OH "   "   31.74 "   31.58 " 31.81 gm. per 100 cc.

The following specific rotations, referred to free cystine, were obtained.   0.2500 gm. of salt dissolved to 25 cc. in H_2O gave $[\alpha]_D^{28} = -90.8°$, $[\alpha]_D^{29} = -89.5°$ (after 6 hours), $[\alpha]_D^{29} = -88.9°$ (after 70 hours), $[\alpha]_D^{29} = -88.9°$ (after 94 hours); 0.2698 gm. of salt dissolved to 25 cc. in CH_3OH gave $[\alpha]_D^{29} = -254°$.   No further readings could be taken as the solution became almost immediately turbid and colored and NH_3 was given off.   On the other hand, even after 94 hours the aqueous solution was perfectly clear and colorless and no NH_3 could be detected.

III. Lithium Salt—To 7.4 gm. of cystine 150 cc. of a 0.40 N

FIG. 3. About × 53.   Second type of K salt crystals

FIG. 2. About × 53.   First type of K salt crystals

FIG. 5. About × 53.   Second type of Li salt crystals

FIG. 4. About × 53.   First type of Li salt crystals

LiOH solution in 60 per cent C_2H_5OH are added. The resulting solution is filtered from excess cystine, 3 volumes of acetonitrile are added, and, after brief mixing, the whole is allowed to stand undisturbed for a few hours. In case of delayed crystallization seeding as mentioned with the Na salt is helpful. A yield of 95 per cent was obtained.

Also in the case of the Li salt the appearance of the crystals varies according to the conditions of crystallization. Figs. 4 and 5 give two typical views. For analysis the material was dried, as described, at 78°.

$C_6H_{10}O_4N_2S_2Li_2$.   Calculated.   Li 5.51
                              Found.   " (volumetric) 5.54, 5.52

The salt is very soluble in water, while the solubilities in C_2H_5OH and CH_3OH are lower than those of the Na and K salts.

C_2H_5OH solution. Found. 0.0075 gm. per 100 cc.
CH_3OH "   "   0.30 " 0.31 gm. per 100 cc.

While all three salts form colorless crystals, the preparations of the Na and K salts usually could not be obtained without a very slight yellow tinge. The Li salt, however, had a pure white appearance. As the analysis also points to a high degree of purity, this salt was chosen for a careful optical study of the stability of the aqueous solution. The determinations were made in a water-jacketed tube with temperature control and the Hg line was used, on account of its greater accuracy. 0.2623 gm. of salt dissolved to 25 cc. in H_2O gave $[\alpha]_{Hg}^{29.06} = -113.5°$, $[\alpha]_{Hg}^{28.64} = -114.6°$, and $[\alpha]_{Hg}^{28.91} = -114.7°$ (after 20 hours); $[\alpha]_{Hg}^{29.18} = -113.7°$ and $[\alpha]_{Hg}^{24.94} = -112.0°$ (after 68 hours); $[\alpha]_D^{29.0} = -94.5°$ (after 68 hours). The two determinations at 29.18° and 24.94° after 68 hours indicate a temperature coefficient of about $+ 0.4°$ $[\alpha]_{Hg}$ for $+ 1°$.

A new preparation of the Li salt, made from cystine of $[\alpha]_{Hg}^{29} = -238.7°$ or $[\alpha]_D^{29} = -202.3°$, corresponding to an l-cystine content of about 99 per cent (8), gave the following rotations. 0.3153 gm. of salt dissolved to 25 cc. in H_2O gave $[\alpha]_{Hg}^{27.9} = -113.9°$, or, corrected, $[\alpha]_{Hg}^{29} = -114.4°$. This salt was dissolved in HCl to produce a solution corresponding to 1 per cent cystine in 1 N HCl. 0.2625 gm. of salt and 13.6 cc. of 2 N HCl diluted to 25 cc. with H_2O gave $[\alpha]_{Hg}^{27.9} = -236.35°$, or, corrected, $[\alpha]_{Hg}^{29} = -233.68°$; $[\alpha]_{Hg}^{27.7} =$

TABLE I

Comparison of the Three Salts Prepared

	Li	Na	K
Alkali solution used..........	0.4 N LiOH in 60 per cent C₂H₅OH	0.46 N NaOH in 82 per cent C₂H₅OH	1 N KOCH₃ in 100 per cent CH₃OH
Precipitant..........	3 volumes CH₃CN	2 volumes CH₃CN	7.5 volumes CH₃CN and ether
Water of crystallization..........	0	1H₂O	0
Alkali in anhydrous salt			
Calculated..........	5.51 per cent	15.23 per cent	24.59 per cent
Found (volumetric)..........	5.54, 5.52 per cent	15.45, 15.54 per cent	24.63, 24.64 per cent
Solubility			
H₂O..........	Very soluble	Very soluble	Very soluble
CH₃OH..........	0.3 gm. per 100 cc.	2.3 gm. per 100 cc.	31.7 gm. per 100 cc.
C₂H₅OH..........	<0.01 " " 100 "	0.16 " " , 100 "	1.9 " " 100 "
Specific rotation [α]ᴅ (calculated for cystine at 29°)			
In H₂O..........	−94.4°	−90.9°	−91.1°
" CH₃OH..........		−257°*	−254°
Stability of solution			
In H₂O..........	Practically stable	Practically stable	Very slow decomposition
" CH₃OH..........		Slow decomposition with formation of NH₃	Rapid decomposition with formation of NH₃

* Correction factor of cystine in HCl used (8).

$-236.80°$, or, corrected, $[\alpha]_{Hg}^{29} = -233.64°$. For comparison, an identical solution was made of the cystine used for the preparation of the salt ($[\alpha]_{Hg}^{29} = -238.7°$), by adding the proper amount of LiOH to the acid solution. 0.2500 gm. of cystine, 0.66 cc. of 3.19 N LiOH, and 13.6 cc. of 2 N HCl diluted to 25 cc. with H_2O gave $[\alpha]_{Hg}^{23.6} = -236.65°$, or, corrected, $[\alpha]_{Hg}^{29} = -235.68°$; $[\alpha]_{Hg}^{29.6} = -234.50°$, or, corrected, $[\alpha]_{Hg}^{29} = -235.96°$. An average difference of $2.16°$ $[\alpha]_{Hg}^{29}$ results between the original cystine and the cystine formed from its Li salt, indicating an almost unchanged recovery of the cystine.

A summarized comparison of the three salts is given in Table I.

SUMMARY

The Li, Na, and K salts of l-cystine were prepared by precipitation of alcoholic alkaline cystine solutions with acetonitrile. The properties of these salts, including their solubilities in H_2O, CH_3OH, and C_2H_5OH, the optical rotation, and the stability of the solutions, were determined.

Note—As this paper was being sent to press we read the publication of Voss and Guttmann (10) on the preparation of alkali salts of amino acids by the use of liquid ammonia. As the authors state that in the case of cystine partial reduction to cysteine interferes with their obtaining a pure disodium salt, we wish to mention that our salts do not give the nitroprusside reaction for sulfhydryl.

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THE EQUILIBRIUM BETWEEN CEREBROSPINAL FLUID AND BLOOD PLASMA

III. THE DISTRIBUTION OF CALCIUM AND PHOSPHORUS BETWEEN CEREBROSPINAL FLUID AND BLOOD SERUM

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The first conclusive experiments demonstrating that calcium does not exist in the serum solely in the form of a simple solution were performed by Rona and Takahashi (1). They found that only 65 per cent of the calcium in the serum was capable of diffusing through a membrane. Cushny (2) obtained similar results when ox serum was filtered through a collodion membrane under 150 mm. mercury pressure. Determinations of the diffusible serum calcium by other investigators (3–5) have varied considerably from those quoted above. Such differences are probably due to the use of different methods. Cameron and Moorhouse (6) first suggested that the cerebrospinal fluid calcium represents the diffusible calcium content of the serum. Except for the studies made by Cameron and Moorhouse (6), Hamilton (7), Pincus and Kramer (8), and Cantarow (9), there are very few data in the literature on the distribution of calcium in the serum and the cerebrospinal fluid. Pincus and Kramer (8) and Hamilton (7) have published the results obtained from a few such determinations.

In our studies of calcium and phosphorus metabolism we have accumulated much data on the distribution of calcium and phosphorus in the serum and cerebrospinal fluid. It was thought advisable to present such of these data as would give us more information concerning the diffusible serum calcium. These studies were made on adult patients[1] admitted to the Medical

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[1] Lumbar punctures were performed in all of these patients for diagnostic purposes, thus enabling us to obtain spinal fluid samples for analysis.

Service of the Massachusetts General Hospital and the Neurological Service of the Boston City Hospital. We are also recording here the results of determinations of the calcium content of the serum, aqueous humor, and cerebrospinal fluid of rabbits and cats.

The majority of the calcium determinations were made after the method described by Fiske and Logan (10) but a few of the earlier values were obtained by the Clark (11) modification of the Kramer-Tisdall method. The two methods were found to give similar values but the Fiske-Logan method yielded more consistent results. Results with this method have been published by Hamilton (7) and by Blackfan and Hamilton (12). The phosphorus determinations were made according to the method of Fiske and Subbarow (13). Serum protein was determined by the Kjeldahl method (14), and protein in the cerebrospinal fluid by the method of Denis and Ayer (15).

We have made simultaneous determinations of the calcium content of human serum and cerebrospinal fluid in 126 instances, and of the phosphorus content in thirty-nine instances. In experimental animals we have determined the serum calcium content in twenty-three instances, with simultaneous cerebrospinal fluid calcium content in eight. In eighteen animals simultaneous determinations were made of the calcium content of the serum and the aqueous humor.

I. Relation of Serum Calcium and Cerebrospinal Fluid Calcium in Normal Individuals

The normal serum calcium content is generally stated to vary from 9 to 11 mg. per 100 cc. (16). While there has been some disagreement in the reported calcium content of the cerebrospinal fluid most of the recent observers, Pincus and Kramer (8), Cantarow (9), Hamilton (7), and others, agree that the normal variation is 4.5 to 5.5 mg. per 100 cc. The results of the above authors are more reliable than those reported by other workers as they made simultaneous determinations on the serum and the cerebrospinal fluid.

Table I shows the simultaneous serum and cerebrospinal fluid

TABLE I

Normal Cases

The calcium content of the serum and cerebrospinal fluid from forty-nine cases without any disease of the central nervous system or any disturbance of calcium metabolism.

Case	Calcium			Case	Calcium		
	Serum	Cerebrospinal fluid	Ratio $\frac{\text{cerebrospinal fluid}}{\text{serum}}$		Serum	Cerebrospinal fluid	Ratio $\frac{\text{cerebrospinal fluid}}{\text{serum}}$
	mg. per 100 cc.	*mg. per 100 cc.*			*mg. per 100 cc.*	*mg. per 100 cc.*	
D. W.	9.48	4.60	0.49	P. P.	10.30	5.05	0.49
A. P.	10.33	5.23	0.51	T. S.	10.20	4.98	0.49
J. G.	9.93	4.88	0.49	A. D. J.	9.90	5.20	0.52
D. L.	10.25	5.05	0.49	D. Y.	9.85	4.96	0.50
R. C.	9.88	5.13	0.52	A. P.	10.10	5.05	0.50
J. F.	10.06	5.01	0.50	M. N.	9.90	5.01	0.51
W. H.	9.65	4.62	0.48	T. D. B.	9.95	5.05	0.51
S. D.	9.35	4.52	0.46	W. U.	10.00	5.10	0.51
E. C.	9.46	4.82	0.51	T. L. A.	10.20	4.90	0.48
F. R.	10.03	5.18	0.51	M. E.	10.15	4.79	0.47
F. R.	10.00	5.20	0.52	A. G.	9.99	5.07	0.51
S. A.	10.06	4.79	0.48	J. W.	10.08	5.11	0.51
A. J.	9.91	5.23	0.53	H. R.	10.14	5.02	0.50
R. O.	10.32	5.16	0.50	C. B.	9.89	4.94	0.50
A. T.	10.08	4.99	0.50	M. G.	9.96	4.99	0.50
M. N.	10.53	4.79	0.45	F. S.	10.16	5.13	0.50
E. G.	10.00	4.90	0.49	W. N.	10.00	5.03	0.50
M. A. C.	10.33	4.93	0.48	D. G.	10.11	5.01	0.50
W. B.	10.10	5.00	0.50	R. B.	9.80	5.05	0.52
F. A.	9.90	4.95	0.50	M. McD.	9.95	4.89	0.50
J. W. A.	9.85	5.10	0.53	W. S. P.	9.97	4.96	0.50
W. S.	10.20	5.07	0.50	B. B.	10.03	5.08	0.51
B. D.	10.15	5.13	0.50	H. T.	10.25	5.01	0.49
R. N.	10.01	4.90	0.49	L. C. E.	10.05	5.09	0.50
M. D.	9.80	5.00	0.51				
Average............................					10.00	5.00	0.50

calcium determinations from forty-nine patients who showed no disease of the central nervous system or other disease in which there is a recognized disturbance of the calcium metabolism. The protein content of the cerebrospinal fluid was normal in every instance and the fluids contained less than five cells per c. mm., thereby eliminating the possibility of increased cerebrospinal fluid calcium due to increased permeability of choroid plexus or meninges.

In this series it will be noted that the serum calcium varied between 9.35 mg. and 10.60 mg. with an average of 10.00 mg., and the calcium content of the cerebrospinal fluid varied between 4.50 and 5.23 mg. per 100 cc., with an average of 5.00 mg.

Mestrezat (17) considers the cerebrospinal fluid to be a dialysate of the serum through the choroid plexus and he showed that the total osmotic pressure, as measured by the freezing point, was identical in the two solutions. This has been confirmed by Teschler (18) and by Thomas (19). The various theories concerning the origin of the cerebrospinal fluid and the reasons for accepting it as a dialysate have been given by Fremont-Smith (20). Cameron and Moorhouse (6) first emphasized the use of the cerebrospinal fluid calcium value as representing the diffusible fraction of the serum calcium.

It should be mentioned that most of the workers who have attempted to determine the amount of diffusible calcium by ultra-filtration or by dialysis have obtained figures somewhat higher than the normal cerebrospinal fluid content. The objections to these procedures are given by Stewart and Percival (16) and they state their reasons why accurate results cannot be expected when such methods are employed. Salvesen and Linder (21) have analyzed protein-free edema fluids in patients with Bright's disease and found 55 to 70 per cent of the serum calcium content in the edema fluid, and they stated that this is the diffusible calcium of the serum. Such edema fluid is derived from abnormal blood plasma in which the calcium equilibrium is probably disturbed.

Updegraff *et al.* (3), however, modified the method of Moritz and obtained values for the diffusible calcium of serum very similar to that of the cerebrospinal fluid. This method was also used by Snell (4) and the results of his experiments in dialyzing serum

against water under 150 mm. mercury pressure coincide very closely with the cerebrospinal fluid calcium. Similar results have also been obtained by Neuhausen and Pincus (5).

Our results as shown in Table I indicate that the cerebrospinal fluid calcium content varies between 45 and 53 per cent of the serum calcium content. In 92 per cent of this series it varied between 48 per cent and 52 per cent with an average of 50 per cent for the entire series. Therefore, if the cerebrospinal fluid calcium can be considered as the diffusible calcium, our results show that approximately 50 per cent of the serum calcium is in a diffusible form. This is in agreement with the figures obtained by Cantarow (9), Pincus and Kramer (8), Lennox and Allen (22), Weston and Howard (23), Hamilton (7), and Cameron and Moorhouse (6).

II. Relation of Serum and Cerebrospinal Fluid Calcium Content to Serum Protein Content

It has been conclusively demonstrated that the serum calcium varies directly with the total serum protein (12, 21, 24, 25). We thought it would be of interest to tabulate our results in the instances where the serum proteins had been determined, in order to ascertain whether the variations in the serum calcium content associated with changes in the total serum protein content were accompanied by changes in the cerebrospinal fluid calcium. For reasons given above only cases with normal cerebrospinal fluid protein were used. Most of these cases, however, had abnormalities of the central nervous system, and in some the serum calcium was outside the normal limits of 9 to 11 mg. per 100 cc. Nevertheless the relation between the calcium content of the serum and spinal fluid closely parallels that found in the normal cases in Table I. The four cases with spinal fluid calcium values of less than 4.40 mg. per 100 cc. are associated with serum calcium values of less than 9 mg. per 100 cc. The summary of Table II shows (although the number of cases in some of the groups is quite small) that there is a tendency for both the serum calcium and the cerebrospinal fluid calcium to vary directly with the serum protein. The ratio of the cerebrospinal fluid calcium to the serum calcium is relatively unchanged and in these thirty-four cases hardly varies from the ratios found in the forty-nine cases in Table I.

TABLE II

Relationship of Serum and Cerebrospinal Fluid Calcium Content to Serum Protein Content

Serum protein	Ca content			Serum protein	Ca content		
	Serum	Cerebrospinal fluid	Ratio $\frac{\text{cerebrospinal fluid}}{\text{serum}}$		Serum	Cerebrospinal fluid	Ratio $\frac{\text{cerebrospinal fluid}}{\text{serum}}$
gm. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*		*gm. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	
5.06	8.80	4.60	0.52	6.56	10.00	5.38	0.54
5.08	9.57	4.42	0.46	6.60	8.90	4.60	0.52
5.41	8.40	3.84	0.46	6.60	9.60	4.87	0.50
5.74	8.70	4.20	0.49	6.62	9.87	4.66	0.47
5.87	9.31	4.80	0.51	6.69	9.00	5.10	0.56
6.07	9.20	4.60	0.50	6.69	9.46	4.66	0.49
6.16	9.60	4.70	0.50	6.82	9.30	4.80	0.51
6.16	8.60	4.60	0.53	6.94	9.80	4.90	0.50
6.13	9 00	4.40	0.49	6.97	9.70	5.30	0.54
6.20	8.40	3.90	0.46	6.98	9.65	4.62	0.48
6.27	9.70	4.70	0.48	7.32	9.35	4.40	0.47
6.29	9.62	5.29	0.55	7.40	10.14	5.30	0.52
6.34	8.90	4.20	0.47	7.60	9.51	4.66	0.49
6.37	10.50	5.20	0.50	7.80	9.48	4.60	0.49
6.41	9.80	5.00	0.51	7.90	10.06	5.01	0.50
6.44	9.70	5.00	0.51	8.20	9.68	4.84	0.50
6.47	9.64	4.84	0.50	8.43	9.55	4.85	0.50

Serum protein	No. of cases	Ca content		Ratio $\frac{\text{cerebrospinal fluid}}{\text{serum}}$
		Serum	Cerebrospinal fluid	
gm. per 100 cc.		*mg. per 100 cc.*	*mg. per 100 cc.*	
5 –5.5	3	8.92	4.29	0.48
5.5–6	2	9.00	4.50	0.50
6 –6.5	12	9.34	4.70	0.50
6.5–7	10	9.52	4.88	0.51
7 –8	5	9.71	4.79	0.49
8 –9	2	9.61	4.84	0.50

III. Relation of Serum and Cerebrospinal Fluid Calcium and
   Phosphorus Content in Non-Suppurative Diseases of the
                Central Nervous System

Table III gives the results of serum and cerebrospinal fluid calcium and phosphorus determinations in thirty patients with various non-suppurative diseases of the central nervous system.

It will be noted that in practically all instances the serum and the cerebrospinal fluid calcium and phosphorus contents were within normal limits.   The ratio of cerebrospinal fluid calcium to the serum calcium was likewise normal.   The protein content of the cerebrospinal fluid of this group showed very little elevation, only four having values of over 100 mg. per 100 cc. and only one case over 200 mg. per 100 cc.   This latter spinal fluid was obtained from a man suffering from myeloma of the sacrum and a toxic polyneuritis.   The total serum protein (5.60 gm. per 100 cc.) and the serum calcium (8.50 mg. per 100 cc.) were low, the cerebrospinal fluid contained 480 mg. of protein and had a calcium content of 4.80 mg., thus giving an abnormally high ratio of cerebrospinal fluid calcium to serum calcium.   The average of these thirty determinations was 9.58 mg. of calcium per 100 cc. for the serum and 4.76 mg. of calcium per 100 cc. for the cerebrospinal fluid with a ratio of cerebrospinal fluid calcium to serum calcium of 50 per cent.

The phosphorus content averaged 4.0 mg. per 100 cc. for the serum and 1.53 mg. per 100 cc. for the cerebrospinal fluid.   The ratio of the serum phosphorus to the cerebrospinal fluid thus being 38 per cent with a range of 31 to 45 per cent.   Hamilton (7) obtained a ratio of 50 per cent in the twenty-six cases he reported. Such results suggest that the protein of the serum influences the diffusion of some of the phosphorus as well as some of the calcium, thus allowing only about half to pass through the human membrane (choroid plexus).

IV. Relation of Serum Calcium and Phosphorus in Suppurative
       Diseases of the Central Nervous System (Meningitis)

Table IV shows the results of twenty-one determinations in patients with meningitis arranged in order of amount of protein in the cerebrospinal fluid.   It will be noted that the serum calcium was low in a number of these patients and some of these had a

TABLE III
Serum and Cerebrospinal Fluid Calcium and Phosphorus Content in Non-Suppurative Diseases of the Central Nervous System

Disease	Protein		Calcium			Phosphorus		
	Serum	Cerebrospinal fluid	Serum	Cerebrospinal fluid	Ratio $\frac{\text{cerebrospinal fluid}}{\text{serum}}$	Serum	Cerebrospinal fluid	Ratio $\frac{\text{cerebrospinal fluid}}{\text{serum}}$
	gm. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.		mg. per 100 cc.	mg. per 100 cc.	
Central nervous system syphilis		105	9.74	4.64	0.47			
" "	6.60	57	9.60	4.87	0.50			
" "	6.07	37	9.20	4.60	0.50	3.70	1.60	0.43
" "	6.82	49	9.30	4.80	0.51	4.70	1.80	0.38
" "	6.16	26	9.60	4.70	0.49			
Multiple sclerosis	8.43	24	9.55	4.85	0.51	4.18	1.30	0.31
" "	6.47	28	9.64	4.84	0.50	4.47		
" "	6.34	22	8.90	4.20	0.47	3.45	1.50	0.43
" "	6.40	132	9.70	4.90	0.50	4.00	1.30	0.32
" "	6.07	69	9.30	4.60	0.50	3.00	1.25	0.42
" "	5.87	34	9.31	4.80	0.51	4.30	1.60	0.37
" "		38	9.60	4.80	0.50	2.90	1.30	0.45
" "		24	9.50	4.50	0.46			
" "		16	10.00	4.50	0.45			
" "	6.94	33	9.80	4.90	0.50	3.90		
" "	6.44	44	9.70	5.00	0.51			
" "	6.27	56	9.70	4.70	0.48			
" "	6.41	29	9.80	5.00	0.51			
Parkinson's	5.08	26	9.57	4.42	0.46			
"	7.32	42	9.35	4.40	0.47			
Subarachnoid hemorrhage	8.20	144	10.20	4.83	0.47			
" "	7.60	236	9.40	4.68	0.49			
" "	7.70	80	9.50	4.60	0.48			
Hematomyelia		80	9.40	4.59	0.48			
Friedreich's ataxia	7.40	63	9.80	4.80	0.50			
Cerebral arteriosclerosis	6.56	26	10.00	5.38	0.54			
Cerebral hemorrhage		98	10.10	5.40	0.53			
Polyneuritis	5.60	480	8.50	4.80	0.56	5.6	2.10	0.37
Diabetes insipidus		29	10.00	5.40	0.54			
Epilepsy		36	9.67	4.38	0.46			
Average			9.58	4.76	0.50	4.00	1.53	0.38

normal serum protein and a normal or low serum phosphorus, which, in accordance with previous observations (12, 21, 24, 25) would indicate a primary disturbance in the calcium metabolism

TABLE IV

Serum and Cerebrospinal Fluid Calcium and Phosphorus Content in Meningitis

Case No.	Protein		Calcium			Phosphorus		
	Serum	Cerebrospinal fluid	Serum	Cerebrospinal fluid	Ratio $\frac{cerebrospinal\ fluid}{serum}$	Serum	Cerebrospinal fluid	Ratio $\frac{cerebrospinal\ fluid}{serum}$
	gm. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.		mg. per 100 cc.	mg. per 100 cc.	
1		21	11.00	5.60	0.51			
2	6.06	32	9.70	5.40	0.56	3.80	1.5	0.40
3	6.47	70	9.05	4.68	0.52	4.32	1.75	0.40
4	6.02	72	8.95	5.00	0.56	3.58		
5	6.61	108	9.20	5.08	0.55	3.70	1.9	0.51
6	5.92	114	10.20	5.10	0.50		1.6	
7	6.76	142	9.50	4.67	0.49	4.08	1.75	0.43
8	6.23	156	8.80	5.10	0.60	3.90	1.5	0.38
9	6.58	162	9.39	5.14	0.55	3.94	2.24	0.56
10	6.50	190	9.05	4.98	0.55	4.49	1.73	0.38
11	7.04	190	9.45	4.83	0.51			
12	7.10	198	9.36	5.06	0.54		1.9	
13	6.17	222	8.77	5.26	0.60	2.50	1.6	0.64
14	6.27	318	9.17	5.35	0.58	3.55	2.3	0.65
15	6.24	320	8.95	5.15	0.60	4.50	1.6	0.35
16	6.30	432	8.94	4.44	0.49	2.73	1.2	0.44
17	5.90	800	8.40	5.04	0.60	3.46		
18	5.91	924	9.40	6.20	0.66	1.40	1.73	1.23
19	4.71	1140	9.40	5.30	0.56	3.70	1.6	0.43
20	5.83	1330	7.80	5.30	0.68		1.9	
21	6.18	1998	8.40	5.52	0.66	2.66	1.8	0.68
Average			9.18	5.15	0.56	3.52	1.72	0.49

in meningitis. The cerebrospinal fluid calcium was relatively high in most of the fluids with the majority of specimens showing a cerebrospinal fluid to serum calcium ratio of over 55 per cent. The

average calcium content for this group was 9.18 mg. per 100 cc. for the serum and 5.15 mg. per 100 cc. for the cerebrospinal fluid and a ratio of 56.1 per cent. Most of the cerebrospinal fluids had a high protein content, and in several the increase in the cerebrospinal fluid calcium can be accounted for by assuming that some of the calcium is associated with the protein of the cerebrospinal fluid. Case 21, for example, had a serum calcium of 8.4 mg. per 100 cc. which under normal conditions should be accompanied by a cerebrospinal fluid calcium of approximately 4.2 mg. The cerebrospinal fluid had 1998 mg. of protein per 100 cc. which is approximately one third of the serum protein. As the non-diffusible calcium (protein-bound calcium) is calculated as 4.2 mg., one-third of this figure would be 1.4. Therefore 4.2 mg., the calculated content of the cerebrospinal fluid, plus 1.4 mg., the calcium bound by the protein, gives 5.6 mg., which is very close to the figure determined—5.52 mg. This relation does not work out as well in all of the cases but the increase in the calcium content of the cerebrospinal fluid in meningitis can probably be accounted for by the abnormal protein present in the fluid and the increased permeability of the meninges and choroid plexus.

The phosphorus content of the serum averaged 3.52 mg. per 100 cc., slightly below the normal average, and the phosphorus content of the cerebrospinal fluid averaged 1.72 mg. per 100 cc. slightly above the normal average. The ratio of cerebrospinal fluid phosphorus to serum phosphorus was 49 per cent in these cases as contrasted with 38 per cent in the cases shown in Table III. The increase in this ratio was probably due, as in the case of the calcium, to the increased protein in the cerebrospinal fluid and the increased permeability of the choroid plexus. This hypothesis is supported by the work of Cohen (26). The increase in the phosphorus ratio in these cases averaged nearly twice as much as the increase in the calcium ratio.

V. Serum: Cerebrospinal Fluid Calcium in Pulmonary Tuberculosis

The serum calcium is thought to be low in acute cases of pulmonary tuberculosis especially those having hemoptysis. Brockbank (27) found the calcium of the serum decreased in the acute cases and increased in the healed cases, but stated that it is not a trustworthy guide of the activity of the pulmonary process because of

the individual variations.   Cantoni (28) and Greisheimer and Van Winkle (29) have also studied the calcium content of the serum in pulmonary tuberculosis.

Table V shows our results in nine active cases of pulmonary tuberculosis.   Five of these cases had a serum calcium content within normal limits, and four were below normal.   Two of the latter cases, however, had a slightly lowered serum protein.   There was no appreciable change in the ratio of the cerebrospinal fluid

TABLE V

Serum and Cerebrospinal Fluid Calcium Content in Pulmonary Tuberculosis

Case No.	Protein		Calcium			Phosphorus	
	Serum	Cerebro-spinal fluid	Serum	Cerebro-spinal fluid	Ratio cerebrospinal fluid serum	Serum	Cerebro-spinal fluid
	gm. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*		*mg. per 100 cc.*	*mg. per 100 cc.*
1	5.72	105	6.75	3.38	0.49		
2	5.41	33	8.40	3.84	0.46		
3	9.10	35	9.50	5.20	0.55		
4	6.37	16	10.50	5.20	0.50		
5	6.97	26	9.70	5.30	0.54		
6	6.29	24	9.62	5.29	0.55		
7	7.04	190	9.45	4.83	0.51		
8	6.60	12	8.90	4.60	0.52		
9	6.16	23	8.60	4.60	0.53	3.9	20
Average................			9.05	4.69	0.51		

to serum calcium content.   It varied from 46 to 55 per cent with an average of 51.7 which was only slightly higher than the normal average.

VI. *Effects of Forced Drainage on the Relation of Serum and Cerebrospinal Fluid Calcium Content*

Kubie (30) in 1928 found that the perivascular spaces of the central nervous system could be drained by draining the cerebrospinal fluid during the intravenous injection of hypotonic solutions. He suggested the use of this method in treating certain diseases

of the central nervous system. His method was modified slightly and used by Fremont-Smith, Putnam, and Cobb (31). They performed a lumbar puncture and drained the cerebrospinal fluid for several hours. While the needle was in the spinal canal the patient was given water by mouth at frequent intervals and 0.25 to 1.0 cc. of vasopressin was injected subcutaneously to prevent diuresis. A specimen of the blood and cerebrospinal fluid were taken for analysis at the beginning and at the end of this procedure.

Table VI shows the figures for the calcium and phosphorus content of the serum and the cerebrospinal fluid in nine of these cases. In eight of the nine cases there was a drop in the calcium content of the serum and of the cerebrospinal fluid, and all but one showed a drop in the serum protein. Most of the changes in the serum and cerebrospinal fluid can be explained on the basis of dilution of the body fluids by the retained water (31).

VII. Serum and Amniotic Fluid Calcium Content

Pregnancy is the one physiological condition in which there is a recognized alteration of serum calcium, and it is presumably due to the drain on the maternal tissues in supplying the demands of the fetus. Various authors have reported a fall in the serum calcium during the latter months of pregnancy. Mazzocco and Bustos Moron (32) found a low serum calcium during pregnancy as did also Bogert and Plass (33), Widdows (34), and Bauer, Albright, and Aub (35). Larson and Fisher (36), working with parathyroidectomized dogs, found that there was a fall in serum calcium during pregnancy although a normal level had been maintained in the animals before they became pregnant.

Very little work has been done on the calcium content of the amniotic fluid and it was thought advisable to report the results obtained in our few cases.

Table VII shows the calcium content of the serum and the amniotic fluid in seven pregnant women at time of delivery. It will be noted that in six of the seven cases the serum calcium was definitely below the limits of normal, with an average of 8.59 mg. per 100 cc. A comparison of the figures with Table II indicates that this change is not due to a loss of serum proteins as they are at the lower level of normal in four of the cases and only slightly below the normal level in the remaining three cases.

TABLE VI

Influence of Water Drinking during the Antidiuretic Effect of Vasopressin on the Serum and Cerebrospinal Fluid Calcium and Phosphorus Content

Case No.	Protein				Calcium						Phosphorus			
	Serum		Cerebrospinal fluid		Serum		Cerebrospinal fluid		Ratio cerebrospinal fluid/serum		Serum		Cerebrospinal fluid	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
	gm. per 100 cc.	gm. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.			mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
1	6.94	7.03	33	27	9.80	9.06	4.90	4.1	0.50	0.45	3.90	4.60	1.20	1.20
2	6.34	6.20	22	11	8.90	8.40	4.20	3.9	0.47	0.46	3.45	3.20	1.50	1.30
3	6.69	6.27	32	56	9.10	9.70	5.05	4.7	0.55	0.49				
4	8.43	6.69	24	21	9.55	9.46	4.85	4.66	0.51	0.49	4.18	3.50	1.30	1.13
5	6.47	6.13	28	23	9.04	9.00	4.84	4.4	0.50	0.49	4.47	3.67		
6	6.40	5.74	132	19	9.70	8.70	4.90	4.2	0.51	0.48	4.00	3.50	1.30	1.00
7	6.07	5.06	69	44	9.30	8.80	4.60	4.6	0.49	0.52	4.00	2.90	1.25	1.20
8	5.87	4.07	34	27	9.31	8.80	4.80	4.6	0.51	0.52	4.30	4.20	1.60	1.50
9			38	16	9.60	9.10	4.80	4.4	0.50	0.48	2.90	3.20	1.30	0.80
Average..	6.65	5.90	57	27	9.54	9.00	4.77	4.40	0.50	0.49	3.77	3.59	1.35	1.16

Case 1 received 2200 cc. of water by mouth during a period of 4 hrs. 55 min.; Case 2, 1800 cc. of water by mouth during a period of 4 hrs. 47 min.; Case 3, 2125 cc. of water by mouth during a period of 5 hrs. 20 min.; Case 4, 600 cc. of 0.5 per cent saline intravenously during a period of 2 hrs. 50 min.; Case 5, 3000 cc. of water by mouth during a period of 3 hrs. 16 min.; Case 6, 3350 cc. of water by mouth during a period of 5 hrs. 43 min.; Case 7, 4020 cc. of water by mouth during a period of 5 hrs. 50 min.; Case 8, 3685 cc. of water by mouth during a period of 4 hrs. 55 min.; Case 9, 3350 cc. of water by mouth during a period of 3 hrs. 20 min.

These cases received from 0.25 to 1.0 cc. of vasopressin subcutaneously preceding the ingestion of water.

The calcium content of the amniotic fluid was found to be be-
tween 5.4 and 8.8 mg. per 100 cc. with an average of 6.59 mg.   It
will be noted that the amniotic fluid had a low protein content,

TABLE VII

Serum and Amniotic Fluid Calcium Content in Pregnancy

Case No.	Protein		Calcium		Phosphorus	
	Serum	Amniotic fluid	Serum	Amniotic fluid	Serum	Amniotic fluid
	gm. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
1	6.36	219	8.76	5.40		
2	6.30	219	8.07	7.44		
3	6.68	120	8.53	7.40		
4	6.20	177	9.58	8.80		
5	5.43	250	8.30	5.75	4.1	0.94
6	5.74	1140	8.60	5.64	5.0	1.20
7	5.53	150	8.30	5.70		
Average............			8.59	6.59	4.5	1.07

TABLE VIII

Effect of Diet on the Serum Calcium Content in Cats

Controls		High Ca diet for 270 days		Low Ca diet		
Animal No.	Serum Ca	Animal No.	Serum Ca	Animal No.	No. of days	Serum Ca
	mg. per 100 cc.		*mg. per 100 cc.*			*mg. per 100 cc.*
401	11.60	337	10.62	338	160	11.42
402	10.06	341	10.02	346	160	10.85
403	9.76	357	12.10	339	160	9.23
404	11.06	360	11.20	345	160	9.48
		362	11.75	342	269	10.50
		364	9.90	343	267	10.25
		356	10.62			
Average..	10.62		10.91			10.28

approximately 200 to 300 mg. per 100 cc., and therefore practically
none of the calcium present in the amniotic fluid was associated
with the presence of protein.   A complete report of other determi-
nations on these fluids will be made later by Dr. A. Makepeace.

VIII. Effect of Diet on Calcium Content of Serum in Cats

In an endeavor to determine whether the calcium content of the diet could influence the serum calcium level, cats were fed a diet containing a known quantity of calcium.

Table VIII shows that the calcium content of the serum of four normal cats averages 10.62 mg. per 100 cc. Seven cats were fed a diet high in calcium for 270 days and the average serum calcium

TABLE IX

Calcium Content of the Serum, Cerebrospinal Fluid, and Aqueous Humor in Cats

Animal No.	Serum	Cerebrospinal fluid	Aqueous humor
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*
337	10.62	6.10	5.63
341	10.00	5.62	6.47
357	12.10	6.25	7.40
360	11.20	6.04	6.10
362	11.75	5.47	6.23
364	9.90	5.41	6.30
356	10.62	5.78	6.28
401	11.60		6.20
402	10.06		5.83
403	9.76		6.71
404	11.06		6.45
342	10.50	5.72	
343	10.25		6.70
351	9.56		6.51
297	10.31		6.49
353	10.02		6.90
314	11.63		7.00
Average.........	10.64	5.79	6.44

content was 10.91 mg. per 100 cc., while the average serum calcium content for six cats fed a diet low in calcium for a period varying from 160 to 269 days was 10.28 mg. per 100 cc.

IX. Calcium Content of Serum, Cerebrospinal Fluid, and Aqueous Humor in Cats

Table IX shows the results of our determinations in seventeen cats.[2] The average calcium content of the fluids was as follows:

[2] All specimens were obtained under ether anesthesia, following which the animals were sacrificed.

serum 10.64 mg. per 100 cc., cerebrospinal fluid 5.79 mg. per 100 cc., aqueous humor 6.44 mg. per 100 cc. From these results it would appear that the serum and cerebrospinal fluid calcium content of cats is slightly higher than in humans, and that the cerebrospinal fluid calcium averages 54 per cent of the serum calcium. The aqueous humor calcium averages 60 per cent of the serum calcium.

SUMMARY

1. The normal serum calcium content was found to vary between 9.35 and 10.6 mg. per 100 cc. with an average of 10.0 mg. per 100 cc. The cerebrospinal fluid calcium ranged from 4.5 to 5.23 mg. per 100 cc. with an average of 5 mg. The ratio of the cerebrospinal fluid calcium to the serum calcium varies from 45 to 53 per cent, with an average of 50 per cent.

2. The serum and cerebrospinal fluid calcium content was found to vary directly with the serum protein in such a way that there was no significant change in the ratio of cerebrospinal fluid calcium to serum calcium with changes in the serum protein.

3. The serum and cerebrospinal fluid calcium content was normal in various non-suppurative diseases of the central nervous system.

4. In meningitis there was a slight diminution of serum calcium content with an increase in the cerebrospinal fluid calcium content. This increase in the cerebrospinal fluid calcium content can probably be accounted for by increased permeability of the meninges and the choroid plexus and the resulting increased protein in the cerebrospinal fluid.

5. There was a slight decrease in the serum and the cerebrospinal fluid calcium content in pulmonary tuberculosis.

6. Water drinking during the antidiuretic action of vasopressin caused a decrease in the serum and cerebrospinal fluid calcium.

7. There was a definite decrease in serum calcium content at the end of pregnancy. The amniotic fluid calcium content was found to vary between 5.4 and 8.8 mg. per 100 cc. with an average of 6.59 mg. per 100 cc.

8. The calcium content of the diet had a slight influence on the level of the serum calcium of cats.

9. In cats the cerebrospinal fluid calcium content averaged 54 per cent of the serum calcium, and the aqueous humor calcium content averaged 60 per cent of the serum calcium.

10. In non-suppurative diseases of the central nervous system, the cerebrospinal fluid phosphorus content was found to vary between 31 and 45 per cent of the serum phosphorus with an average of 38 per cent. In suppurative diseases of the nervous system, the ratio of cerebrospinal fluid phosphorus to serum phosphorus varied between 35 and 123 per cent with an average of 40 per cent.

One of us (H. H. M.) takes this opportunity to thank Dr. Frank Fremont-Smith for his aid and guidance in this work, and for the data on serum and cerebrospinal fluid protein.

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THE EQUILIBRIUM BETWEEN CEREBROSPINAL FLUID AND BLOOD PLASMA

IV. THE CALCIUM CONTENT OF SERUM, CEREBROSPINAL FLUID, AND AQUEOUS HUMOR AT DIFFERENT LEVELS OF PARATHYROID ACTIVITY

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The administration of an active parathyroid extract causes the serum phosphorus to fall and the serum calcium to rise (1–4). Although the effect of parathyroid injections on the serum calcium and serum phosphorus is very marked, the serum proteins remain unchanged (3); with normal parathyroid activity the serum calcium varies directly with the serum protein (5, 6). Merritt and Bauer (7) have observed a similar relationship between the calcium of the cerebrospinal fluid and the serum protein. The variations of the serum calcium at different levels of parathyroid activity have been thoroughly studied but very little is known concerning the calcium content of other body fluids in these various states. It is our purpose to report the relation of the serum calcium to cerebrospinal fluid and aqueous humor calcium at varying levels of parathyroid activity. The methods used have been previously described (7).

I. Hyperfunction of Parathyroid Glands

A. In Man—Hyperfunction of the parathyroid glands results in a rise in the serum calcium content, but there is very little information as to whether this increase is mainly or wholly in the diffusible or the non-diffusible fraction of the serum calcium. Moritz (8) working with rabbits found an increase in both frac-

tions in six animals when parathyroid extract was administered. In three instances he observed a relatively greater increase in the diffusible fraction. Stewart and Percival (9) claim that the calcium mobilized by the parathyroid glands is largely diffusible calcium. They stated that 58 to 80 per cent of the serum calcium is normally diffusible. With the administration of a parathyroid extract this increases to 80 or 90 per cent. Liu (10) found a
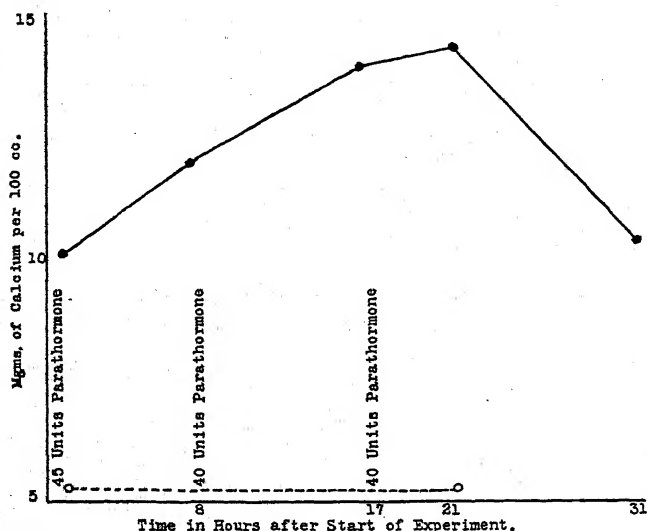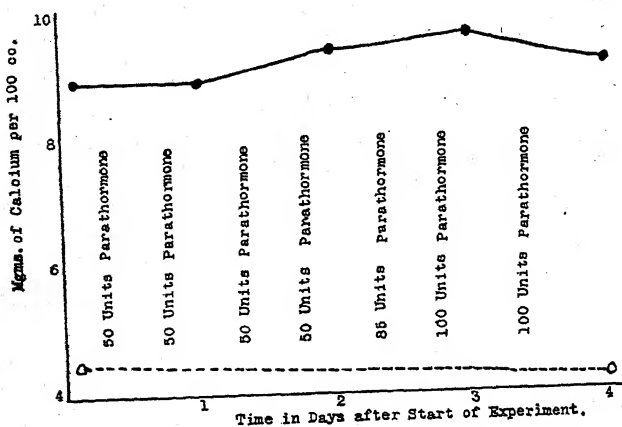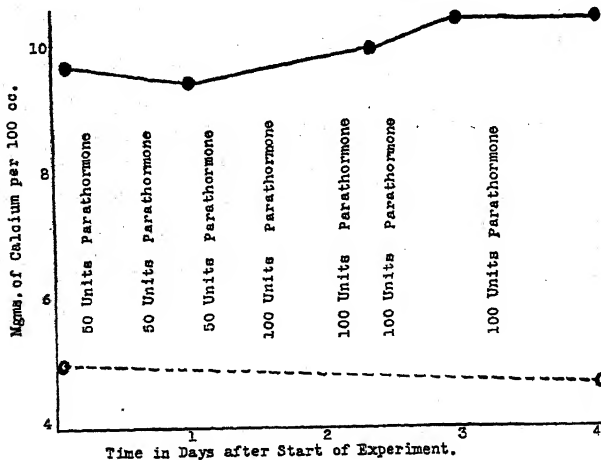

CHART I. The calcium content of the serum and cerebrospinal fluid of a 16 year old male with epilepsy, before and after parathormone injections. The solid line represents the serum calcium, the broken line the cerebrospinal fluid calcium.

relatively greater rise in the diffusible fraction in two patients with parathyroid tetany when they were given parathyroid extract.

On the other hand Snell (11) reported that 45 to 60 per cent of the serum calcium is diffusible and that there is very little, if any, change in this ratio with dysfunction of the parathyroid glands.

For reasons previously stated (7) we have considered the cerebrospinal fluid calcium content to be a more accurate physiological representation of the diffusible calcium of the serum than the values obtained by the various filtration methods used to deter-

mine diffusible serum calcium. Cantarow (12), also working on this hypothesis, found in man that the cerebrospinal fluid calcium rose 3 to 4 hours after the giving of 40 units of parathyroid extract, although the serum calcium content had remained unchanged. At the end of 12 hours, however, the cerebrospinal fluid calcium had in most instances returned to or fallen below its original level whereas the serum calcium content was then at its height. Berencsy (13) reported a rise in the cerebrospinal fluid calcium content
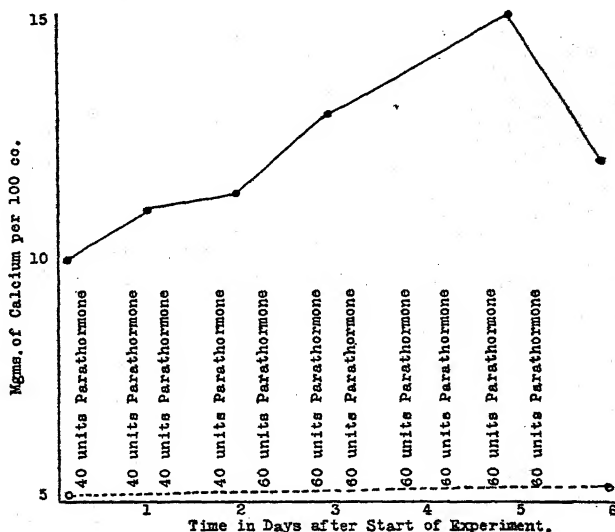
CHART II. The calcium content of the serum and cerebrospinal fluid of a 15 year old male with epilepsy, before and after parathormone injections. The solid line represents the serum calcium, the broken line the cerebrospinal fluid calcium.

on giving 1 unit of parathormone per kilo per day for 2 days, but his values for normal cerebrospinal fluid calcium varied from 6.10 to 8.41 mg. per 100 cc., which are outside of the range commonly accepted as normal. Therefore his results are difficult to interpret.

The results of the subcutaneous administration of parathormone (Lilly) to seven patients are shown in Charts I to VII. It will be noted that in five of the seven cases a substantial rise in the serum calcium content was obtained. In two cases, Charts VI and VII,
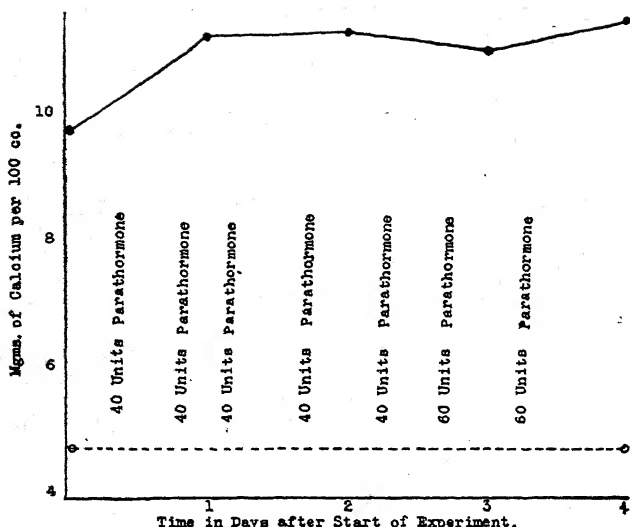
CHART III. The calcium content of the serum and cerebrospinal fluid of a 26 year old male with epilepsy, before and after parathormone injections. The solid line represents the serum calcium, the broken line the cerebrospinal fluid calcium.
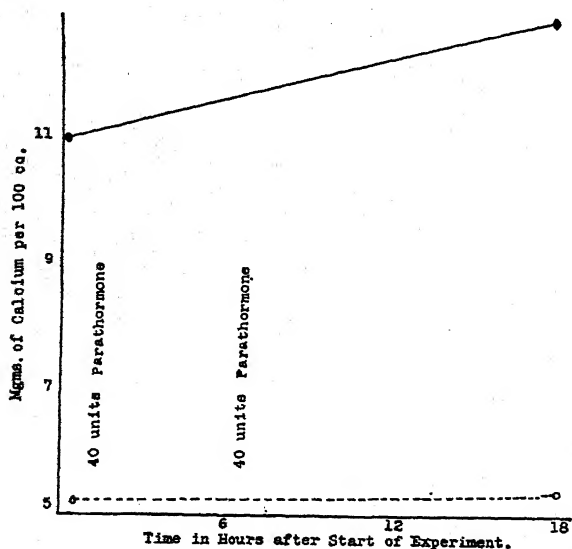
CHART IV. The calcium content of the serum and cerebrospinal fluid of a 16 year old female convalescing from meningococcus meningitis, before and after parathormone injections. The solid line represents the serum calcium, the broken line the cerebrospinal fluid calcium.

no significant elevation in the serum calcium content was observed although larger doses of the same parathyroid extract which had been effective in the other five cases were used.   In these two cases a slight fall in the cerebrospinal fluid calcium occurred.   In none of the seven patients was any elevation of the cerebrospinal fluid calcium noted.   In each instance the ratio of the cerebro-
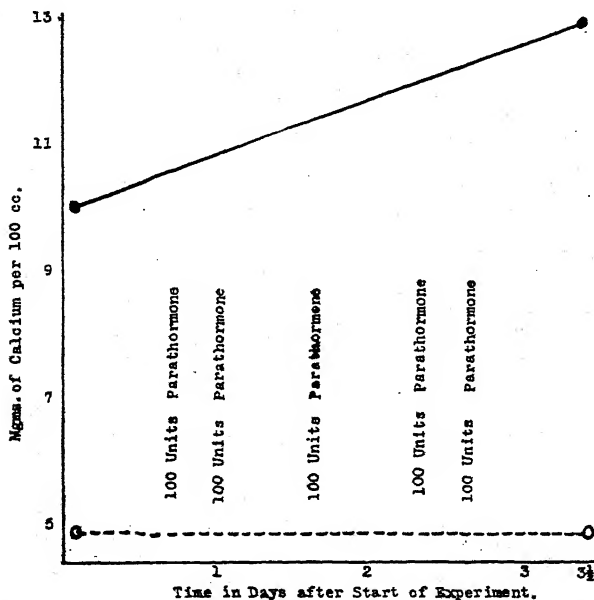
CHART V. The calcium content of the serum and cerebrospinal fluid of a 56 year old male with idiopathic parathyroid tetany, before and after the daily administration of 100 units of parathormone.   The solid line represents the serum calcium, the broken line the cerebrospinal fluid calcium.

spinal fluid calcium content to the serum calcium content was reduced.

In Case 2 (Chart II) the serum calcium content was maintained above normal for 6 days but no change in the cerebrospinal fluid calcium content was noted at the end of this period.

B. *In Animals*—Charts VIII and IX show the results obtained in two cats.   The serum and cerebrospinal fluid calcium content was determined and then the animals were given 100 units of

CHART VI. The calcium content of the serum and cerebrospinal fluid of a 56 year old male with syphilis of the central nervous system, before and after parathormone injections. The solid line represents the serum calcium, the broken line the cerebrospinal fluid calcium.

CHART VII. The calcium content of the serum and cerebrospinal fluid of a 43 year old male with epilepsy, before and after parathormone injections. The solid line represents the serum calcium, the broken line the cerebrospinal fluid calcium.

parathormone. In these animals the serum and cerebrospinal fluid were withdrawn 3 hours and 24 hours following the injection of the parathormone. It will be noted that there was no rise in the serum calcium content 3 hours after the injection but there was a definite elevation in the cerebrospinal fluid calcium content. At the end of 24 hours the serum calcium had risen about 2 mg. and the cerebrospinal fluid calcium had returned to the preinjec-
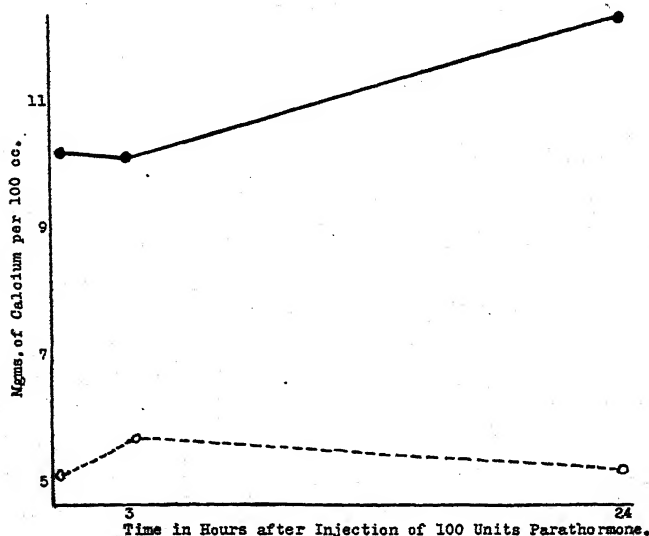
CHART VIII. The calcium content of the serum and cerebrospinal fluid of Cat 3, before, 3 hours after, and 24 hours after the injection of 100 units of parathormone. The solid line represents the serum calcium, the broken line the cerebrospinal fluid calcium.

tion level. These results are in accord with the findings of Cantarow (12).

In an endeavor to determine the effect of parathormone administration on the bone trabeculæ, daily injections were given to a litter of rabbits for a period of 91 days. The extract was given in increasing dosages until each animal was receiving 8 units per day. This dose they received for 56 to 74 days of the period. At the end of this period (24 hours after the last injection) the animals were killed by etherization. The results of the experi-
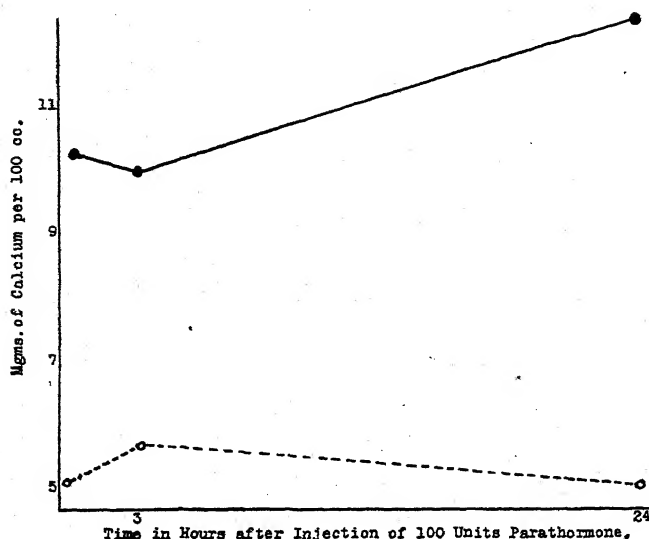
CHART IX. The calcium content of the serum and cerebrospinal fluid of Cat 4, before, 3 hours after, and 24 hours after the injection of 100 units of parathormone.   The solid line represents the serum calcium, the broken line the cerebrospinal fluid calcium.

TABLE I

Effect of Prolonged Parathormone Injections (91 days) on the Serum and Aqueous Humor Calcium Content in Rabbits

	Controls			Injected animals	
	Ca content				Ca content	
Animal No.	Serum	Aqueous humor	Animal No.	Serum	Aqueous humor
	mg. per 100 cc.	*mg. per 100 cc.*		*mg. per 100 cc.*	*mg. per 100 cc.*
351	9.56	6.51	301	15.5	7.70
297	10.31	6.49	298	14.2	6.17
353	10.02	6.90	349	14.6	8.44
314	11.63	7.00	350	15.75	7.33
			352	13.2	6.96
Average....	10.38	6.72		14.65	7.32

ments have been previously reported by one of us (W. B. (14)). The serum and the aqueous humor were withdrawn just before the animals were killed, and the results of the calcium determinations on these fluids are reported herewith. Table I shows that the serum calcium content for four normal control rabbits averaged 10.38 mg. per 100 cc. and the aqueous humor calcium content averaged 6.72 mg. per 100 cc. The average values for the five injected animals were 14.65 mg. of calcium per 100 cc. for the serum and 7.32 mg. per 100 cc. for the aqueous humor. This gives an average increase of 4.27 mg. for the serum and only 0.6 mg. increase for the aqueous humor. From these results it would appear that the injection of parathyroid extract causes only a slight increase in the calcium content of the aqueous humor. The relatively high calcium content of the aqueous humor of the rabbit as compared to that of the cerebrospinal fluid of man or cat is possibly explained by the fact that when aqueous humor is removed the last portion of the sample usually contains a large excess of protein and therefore approaches the serum in composition.

II. *Hypofunction of Parathyroid Glands*

It has been consistently observed that with hypofunction of the parathyroid glands there is a decrease in the serum calcium content, but there has been some disagreement as to what portion of the serum calcium is most affected. Salvesen and Linder (15) removed the parathyroid glands from two dogs and obtained a fall in the serum calcium content without any change in the serum proteins, so they concluded that the calcium loss must be from the non-protein bound, or diffusible portion. Moritz (8) working with parathyroidectomized rabbits found by diffusion experiments that the decrease of the calcium content was in both portions, but in six of his seven animals the decrease was relatively greater in the diffusible portion. Von Meysenbug and McCann (16) observed in two cases of human rickets and four cases of parathyroid tetany in dogs that the total calcium content of the serum was much reduced, but the portion of the calcium diffusible through a collodion membrane was 60 to 70 per cent of the total amount, which was the percentage that they had obtained for normal dog and human sera. Cruickshank (17) reported that 60

to 70 per cent of the serum calcium would normally diffuse through a collodion membrane, but in severe parathyroid tetany the percentage of diffusible calcium increased to as much as 94 per cent of the total. He postulated that in parathyroid tetany there was a rapid breakdown of the protein-bound calcium in order to supply the tissue demands for calcium. Cameron and Moorhouse (18), considering the cerebrospinal fluid calcium as representing the diffusible calcium, found in normal dogs that the cerebrospinal fluid calcium equalled 53 per cent of the serum calcium; whereas in the parathyroidectomized animals the cerebrospinal fluid

TABLE II

Serum and Cerebrospinal Fluid Calcium and Phosphorus Content in Parathyroid Tetany

Case	Ca content			P content		
	Serum	Cerebro-spinal fluid	Ratio, cerebrospinal fluid: serum	Serum	Cerebro-spinal fluid	Ratio, cerebrospinal fluid: serum
	mg. per 100 cc.	*mg. per 100 cc.*		*mg. per 100 cc.*	*mg. per 100 cc.*	
K. L.	8.20	4.42	0.54			
B. W.	4.50	4.55	1.00	6.15	1.95	0.31
B. W.*	6.70	4.40	0.65	4.40	1.81	0.41
H. T.	5.93	5.18	0.87			

* After receiving 100 units of parathormone each day for 3 days.

calcium, while decreased in absolute amount, equalled 70 to 100 per cent of the serum calcium.

Table II shows the results of our determinations of the serum and cerebrospinal fluid calcium contents in three cases of human parathyroid tetany. In these cases there was a marked reduction of the serum calcium content with only a very slight or no decrease of the cerebrospinal fluid calcium content, and the ratio of cerebrospinal fluid to serum calcium was markedly increased reaching 100 per cent in one instance.

Table III shows the values for the serum and cerebrospinal fluid calcium contents in all of the parathormone experiments, before and after the injections. Before the injections the ratio of cerebrospinal fluid calcium to serum calcium varied from 49 to 53 per

cent with an average of 51 per cent. After the injections the ratio varied from 37 to 49 per cent with an average of 42 per cent. If the cerebrospinal fluid calcium is considered as the diffusible calcium it would seem that parathormone affects only the nondiffusible portion. Table III also shows the values for the phosphorus content of the serum and the cerebrospinal fluid. Although there were individual variations there tended to be a decrease in both the serum and cerebrospinal fluid phosphorus

TABLE III

Serum and Cerebrospinal Fluid Calcium Content before and after Parathormone Injections

Case No.	Total units parathormone	Interval	Serum Ca		Cerebrospinal fluid Ca		Ratio, cerebrospinal fluid Ca: serum Ca		Serum P		Cerebrospinal fluid P	
			Before	After	Before	After	Before	After	Before	After	Before	After
			mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*			*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
1	125	21 hrs.	10.14	14.14	5.30	5.40	0.52	0.38	5.30	5.90	2.00	1.6
2	580	6 days	10.00	12.01	5.00	5.20	0.50	0.43	5.10	3.50	1.60	1.4
3	320	4 "	9.80	11.30	4.80	4.74	0.49	0.42	4.10	3.30	1.70	1.5
4	80	20 hrs.	11.00	12.90	5.60	5.20	0.51	0.40				
5	300	3½ days	10.15	12.80	4.95	4.70	0.49	0.37	4.27	4.40	1.65	
6	485	3½ "	9.20	8.80	4.90	4.30	0.53	0.49	4.30	4.60	1.70	1.5
7	550	3½ "	·9.90	10.20	5.10	4.80	0.51	0.47	3.90			1.5
8	100	24 hrs.	10.25	12.50	5.24	5.30	0.51	0.42				
9	100	24 "	10.30	12.20	5.35	5.38	0.52	0.44				
Average.......			10.08	11.87	5.13	5.00	0.51	0.42	4.48	4.42	1.73	1.50

content with a rise in the calcium content. This occurred also in one of the cases of parathyroid tetany when given parathormone (B. W. in Table II).

Chart X shows the serum and cerebrospinal fluid calcium content in the cases of parathyroid tetany and in the cases with high serum calcium resulting from parathormone injections as compared with forty-nine normal cases. This chart shows that the parathyroid hormone has only a slight influence on the cerebrospinal fluid calcium content. The serum calcium content ranged from 4.5 mg.

to 14.1 mg. per 100 cc. while the cerebrospinal fluid calcium content was within the normal range of 4.5 to 5.5 mg. per 100 cc.

The Influence of the Parathyroid Glands on the Serum and Cerebrospinal Fluid
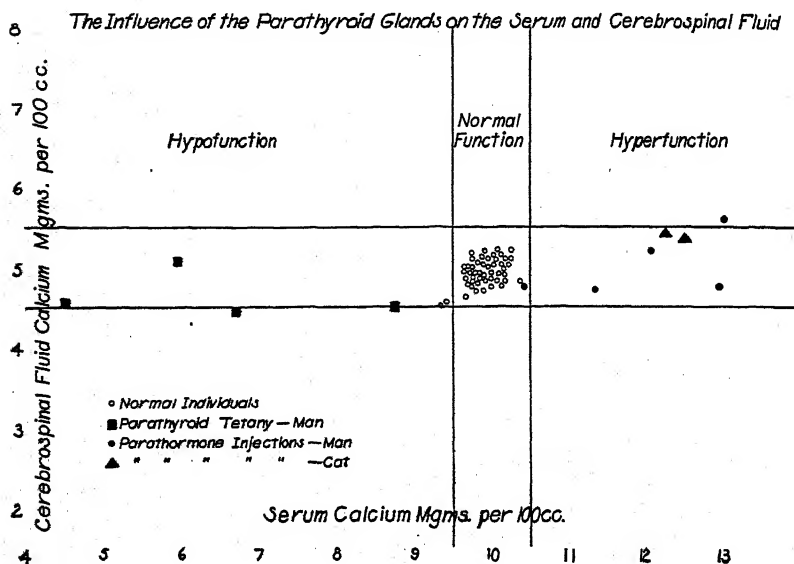
CHART X. Graphic presentation of data contained in Table I of the preceding paper and Tables II and III of this paper.   Each point on the graph represents the simultaneous determination of calcium both in serum and in cerebrospinal fluid.   In these experiments, although the serum calcium varied from 4.5 mg. per 100 cc. to 13.9 mg. per 100 cc., the cerebrospinal fluid calcium remained within the range of 4.45 mg. per 100 cc. to 5.6 mg. per 100 cc.

SUMMARY

The constancy of the serum calcium level needs no comment. The constancy of the cerebrospinal fluid calcium level is more remarkable.   The serum calcium varies with serum proteins, low serum proteins being regularly associated with low serum calcium. The variations in serum calcium which accompany changes in parathyroid activity are, however, independent of the serum protein level (3).   It is interesting therefore to compare the effect upon the cerebrospinal fluid calcium level of these two apparently different types of variation in the serum calcium.   The outstanding result in both cases is that the cerebrospinal fluid

calcium remains remarkably constant in spite of well marked changes in the serum calcium level. The striking lowering of the serum calcium occurring in parathyroid tetany and the sustained elevation of the serum calcium following parathormone administration were without appreciable effect upon the cerebrospinal fluid calcium level. Variations in serum calcium associated with different levels of serum protein were reflected to a slight degree in the cerebrospinal fluid. Our data (7), however, were not sufficient to bring out this relationship clearly. We observed the greatest variation in the cerebrospinal fluid calcium in patients who took water by mouth during the antidiuretic action of vasopressin (7). In these cases we noted a dilution of both the blood serum and the cerebrospinal fluid (19).

If we accept the cerebrospinal fluid as a dialysate in osmotic and hydrostatic equilibrium with the blood plasma, our data would indicate that variations in serum calcium occurring during parathyroid tetany or following the administration of parathyroid gland extract are chiefly and perhaps wholly limited to the non-diffusible calcium. Our data would also suggest that those variations in serum calcium which are associated with different levels of serum protein involve both the diffusible and the non-diffusible calcium.[1]

[1] Since this was written an important paper has appeared (Morgulis, S., and Perley, A. M., *J. Biol. Chem.*, 88, 169 (1930)). In dogs the marked increase in serum calcium, produced by feeding calcium in conjunction with injections of parathyroid extract, or by prolonged intravenous injections of calcium salts, was associated with but minor increases in the cerebrospinal fluid calcium. Similarly the marked fall in serum calcium following parathyroidectomy was associated with but minor decreases in the cerebrospinal fluid calcium. In one instance, however, a serum calcium of 4.0 mg. per 100 cc. was accompanied by a cerebrospinal fluid calcium of 3.7 mg. per 100 cc. With the exception of this case the lowest cerebrospinal fluid calcium after parathyroidectomy was 4.6 mg. per 100 cc., while the highest cerebrospinal fluid calcium after parathyroid extract injection was 6.5 mg. per 100 cc. These figures are only slightly outside their range for normal dogs—5.2 to 5.9 mg. per 100 cc. It is interesting that the greatest rise in cerebrospinal fluid calcium was obtained after intravenous injection of calcium salts—5.6 to 8.3 mg. per 100 cc. Our greatest fall in cerebrospinal fluid calcium occurred after water drinking during the antidiuretic action of posterior lobe pituitary extract—4.9 to 4.1 mg. per 100 cc. (7). Their results as well as ours emphasize the remarkable constancy of the cerebrospinal fluid calcium level.

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NOTE ON THE PREPARATION OF BROMOACETYL SUGARS AND OF ACETOGLUCALS

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The present paper contains a description of modifications in the procedures for the preparation of bromoacetyl derivatives of sugars and of their acetoglucals (the term glucal is used in its generic sense). The procedures described have been successfully used in this laboratory for some time.

I. Preparation of Bromoacetyl Derivatives—The usual procedure in the preparation of the bromoacetyl sugar consists in the following steps: (1) Formation of the bromoacetyl derivative either directly from the sugar with hydrogen bromide and acetic anhydride or from the pentacetate with hydrogen bromide in acetic acid; (2) extraction of the bromoacetyl derivative from the reaction mixture with chloroform; (3) removal of the excess of hydrobromic acid, acetic anhydride, and acetic acid by washing the chloroform solution of the reaction product with bicarbonate solution and then with water; (4) drying of the chloroform solution; (5) concentration of the chloroform solution and crystallization of the product.

We find that steps (2), (3), and (4) can be entirely eliminated if the reaction product resulting in step (1), is taken up in toluene and the solution is concentrated under reduced pressure (temperature of the water bath 40–50°), the toluene being renewed until all the excess reagents are removed. The bromoacetyl derivative generally crystallizes directly in the distilling flask.

II. Preparation of Acetoglucals—In the preparation of acetoglucals from the corresponding bromoacetyl sugars, the number of steps can also be reduced and the procedure simplified by the use of toluene. In fact, in this case the use of toluene is particularly

indicated, as it forms an azeotropic mixture with the acetic acid which is used as solvent when treating the bromoacetyl sugar with zinc dust.

<div align="center">EXPERIMENTAL</div>

Preparation of Bromoacetylmonoses

For the success of the operation it is essential to have a perfectly pure, anhydrous, finely pulverized sugar. Dry hydrogen bromide is passed into acetic anhydride until the latter contains 40 gm. of the gas per 100 gm. For each 50 gm. of the sugar 250 cc. of the reagent are required. The sugar is divided into 10 approximately equal portions, each kept in a stoppered test-tube. The acetic anhydride containing the hydrobromic acid is cooled to 10° and into this solution one portion of the sugar is added under constant shaking. As the sugar dissolves, the temperature of the solution rises, but by cooling the solution in ice water, the temperature is not allowed to exceed 30°. The first portion of the sugar dissolves rather slowly but the subsequent portions dissolve much more readily. Before adding a new portion of the sugar, the solution is again cooled to 10°. After all the sugar has been introduced, the solution is immersed in an ice-salt mixture and cooled to 0°. Hydrogen bromide is passed into the solution until the total content is 60 gm. per 100 gm. of acetic anhydride. The solution, which should be only a very light straw-yellow, is then allowed to stand for 1 hour, after which it is concentrated under reduced pressure to half its original volume. 300 cc. of toluene are added and the distillation is continued until the residue has become a thick syrup. Three additional portions of toluene are added and removed by distillation. In order to obtain a maximum yield, it is important that all reagents be removed as completely as possible. When this is accomplished, the bromoacetyl sugar crystallizes in the distilling flask. In order to obtain a perfectly colorless product on first crystallization, the substance is dissolved in a minimum quantity of warm ether and the ethereal solution is decolorized with charcoal and filtered. Bromoacetylglucose generally crystallizes directly out of ether but when this is not the case, crystallization may be effected by the addition of a small portion of low boiling petrolic ether (35–40°).

In the cases of xylose and arabinose, good results were also

obtained when the sugar was suspended in cold acetic anhydride and hydrobromic acid was passed into the suspension until 60 gm. of the gas were absorbed per 100 gm. of acetic anhydride, care being taken not to allow the temperature to rise above 30°. As soon as all the sugar had dissolved, the solution was diluted with toluene and treated as above. Under these conditions, the solution of the sugar proceeds at a slower rate than in the former procedure but the yield is identical.

The preparation of 1-bromotetracetyl-d-mannose needs special mention. This substance had been generally obtained in the form of a syrup. In 1926 Levene and Sobotka[1] succeeded in obtaining it in the form of crystals imbedded in a syrupy mass. Very recently, Micheel and Micheel[2] described the preparation of the substance in crystalline form. Their procedure was the same as that described by Levene and Sobotka and the specific rotation of their substance was identical with that described by Levene and Sobotka. Apparently the publication of these authors remained unknown to Micheel and Micheel. The latter remarked that due to the low tendency of the substance to crystallize, its complete purification was a very difficult matter. When prepared by the toluene method, the substance is so free of impurities that recrystallization offers no difficulty. Thus far, however, we have prepared the crystalline bromoacetylmannose only from the β-pentacetate and not from the free sugar.

The yields were as follows:

	Bromoacetyl derivative gm.
50 gm. glucose	85
50 " β-mannose pentacetate	32
10 " xylose	9.0
10 " arabinose	9.0–9.5

Preparation of Acetylglucals

The treatment of the bromoacetyl sugar with zinc dust was performed under the original conditions of Fischer and Zach,[3]

[1] Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, 67, 774 (1926).

[2] Micheel, F., and Micheel, H., *Ber. chem. Ges.*, 63, 390 (1929).

[3] Fischer, E., and Zach, K., *Sitzungsber. k. preuss. Akad. Wissensch.*, 16, 311 (1913).

the only difference being that the quantity of zinc dust employed by us is one-half of that used by Fischer and Zach.   After filtering off the zinc dust, the reaction product is extracted with toluene until further extracts and also the aqueous residue no longer absorb bromine.   The toluene solution is concentrated under reduced pressure, the toluene is renewed, and the distillation continued until all acetic acid is removed.   The residue is then taken up once or twice in a little benzene and the distillation continued. This residue is taken up in ether, from which the acetylglucal generally crystallizes on cooling and scratching.   If the crystallization does not proceed rapidly, it is facilitated by the addition of a little of low boiling petrolic ether.   The average yield of once recrystallized triacetylglucal is 25 gm. from 50 gm. of bromoacetylglucose.

The triacetylglucal from mannose[4] and the diacetyl-3-methylglucal[5] described in previous papers were also prepared by this method.

[4] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **90**, 89 (1931).

[5] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **88**, 513 (1930).

THE MECHANISM OF IRON CATALYSIS IN CERTAIN OXIDATIONS

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It is well known that ferrous iron is comparatively stable in acid solution and that it is rapidly oxidized to the ferric state by the oxygen of the air in alkaline solution. No satisfactory explanation for this has been brought forward. One difference in the condition of the iron in the two solutions immediately suggests itself. This is that in the acid solution the iron exists as ferrous ion while in the alkaline solution it exists as unionized ferrous hydroxide. If we remember that the oxidation of ferrous iron consists of the loss of one electron from the kernel of the iron atom, we might expect that ionized and unionized ferrous iron would differ in the ease with which they may be oxidized. Thus, the oxidation of ferrous ion would involve the separation of a negative charge from a kernel that is already carrying two positive charges. This would be comparatively difficult. On the other hand, unionized ferrous hydroxide should be more readily oxidized for here it is only necessary to separate an electron from a previously electrically neutral substance. With this consideration to guide us we would expect that if ferrous iron can be obtained in an unionized compound it will be readily oxidized regardless of the acidity of the solution.

In a previous communication Smythe and Schmidt (1) have reported studies on the compounds which form very slightly ionized complexes with ferric ion. If the suggestions developed by them concerning the manner in which these complexes are formed are correct, then these same compounds should form ferrous complexes and, in accordance with the above considerations, the oxidation of the iron contained in the complexes should depend

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upon the acidity of the solution only in so far as the stability of the complex depends upon this acidity. We would expect then that in any given solution the rate of oxidation would be proportional to the concentration of the ferrous complex. The concentration of this complex is determined by the concentrations of the ferrous ions and the complex-forming anions. The concentration of the anions is, within a certain pH range, determined by the pH of the solution; so we would expect the rate of oxidation within this pH range to vary as the pH is varied.
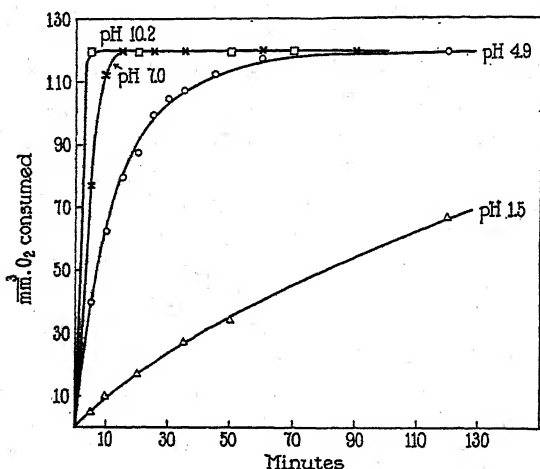
FIG. 1. Each flask contained 0.8 cc. of 0.1 M pyrophosphate solution and 0.2 cc. of 0.1 M FeSO₄. The O₂ equivalent of the iron present is 120 c.mm.

An excellent substance to test this hypothesis is pyrophosphoric acid. This gives a ferrous complex which is stable over a wide pH range, and the ferrous iron is the only substance in the solution that can be oxidized. Experiments at various pH values have been carried out with this acid and the results are shown in Fig. 1. It is readily seen that they bear out the above predictions.

The experiments were carried out in the Warburg respiration apparatus. Brodie's solution was used as manometer fluid. The temperature was maintained at 25°. The total volume of the flask was about 25 cc. and 1 cc. of liquid was used. To get reproducible results it is necessary to use the same or very similar vessels. Ves-

sels of different shape allow different degrees of shaking and this affects the rate of oxidation. Only a few minutes are required for the solutions to come to equilibrium before mixing. A similar flask without any iron was run as a control thermobarometer.

The pyrophosphoric acid solutions were made by dissolving crystals of $Na_4P_2O_7 \cdot 10\ H_2O$ in water and adjusting the pH by the addition of hydrochloric acid. The pH values recorded were determined by the hydrogen electrode and refer to the pyrophosphate solution before the addition of the ferrous sulfate. The
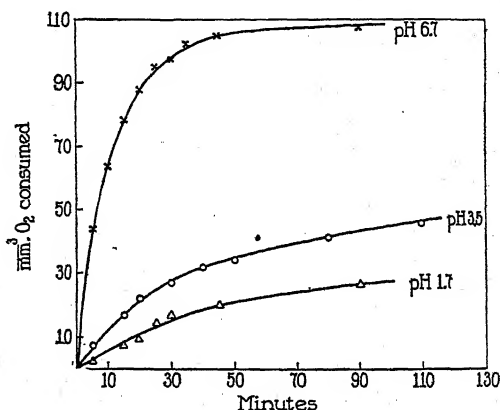
FIG. 2. Each flask contained 0.8 cc. of 0.2 M metaphosphoric acid solution and 0.2 cc. of 0.1 M $FeSO_4$. The O_2 equivalent of the iron present is 120 c.mm.

addition of the iron salt would change the pH some, but the solutions are certainly in the correct order.

Experiments with metaphosphoric acid have been carried out in the same way and the results are shown in Fig. 2. In general the results are the same as those with pyrophosphoric acid, but it may be seen that the rate of oxidation at any one pH is not the same in the two cases. This is to be expected from the above considerations since the structure and the dissociation constants of the two acids are not the same. The metaphosphoric acid solutions were made by dissolving the free acid in water and adjusting the pH by the addition of sodium hydroxide. When ferrous sulfate was brought to pH 2.0 by means of sulfuric acid instead of

metaphosphoric acid, no oxidation was observed within a comparable length of time.

Perhaps the best known ferrous complex is the ferrocyanide. The iron in this complex is certainly not ferrous ion and yet its oxidation by the oxygen of the air is exceedingly slow (2, 3). The methods used by the above authors in studying this are open to severe objection and this compound needs special investigation. In any case, in line with the reasoning of this paper, one would expect that the iron in this compound would not be readily oxidized, for if we consider the structure of the ferrocyanide ion to be

$$\left[\begin{array}{ccc} CN & & CN \\ CN & Fe & CN \\ CN & & CN \end{array}\right]^{\equiv}$$
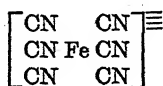
we can see that the iron atom is surrounded by four negative charges. In order for an electron to escape from the iron it must penetrate this negative atmosphere and this is not easy to do. If one could repress the ionization of these cyanide groups so they would exist as CNH instead of as CN^-, it should then be easier for the iron to be oxidized. This repression is very difficult, however, for ferrocyanic acid is a strong acid. Kolthoff (3) has estimated that its fourth constant is 5×10^{-4}. The third one was too strong for him to estimate. It is possible to reduce this negative charge by substituting an NH_3 group for one of the CN^- groups. The iron of this substance, pentacyanoammine-ferroate, is oxidized by molecular oxygen at an appreciable rate (4). Baudisch and Davidson (5) found the oxygen consumption to be much more rapid at a pH of 2.0 than at pH 7.0 or 12.0. It is striking that here increase of pH slows down the rate of oxidation in contrast to the former cases. They interpreted this in terms of the following equation, $H^+ + \frac{1}{2}O_2$ (dissolved) $+ 2[Fe(CN)_5 \cdot NH_3]^{\equiv} \rightleftarrows OH^- + 2[Fe(CN)_5NH_3]^{=}$, which shows the hydrogen ion taking part in the reaction. In accord with our other considerations we would regard the influence of the increased hydrogen ion concentration as due to a repression of the ionization and consequent decrease of the negative charge on the iron compound.

We know that the hydroxy organic acids are good complex formers (1) so they should unite with ferrous iron and make it readily oxidizable. The results obtained with these acids are more complicated, however, for the organic radical is also oxidized

and we are led into a consideration of catalysis by iron. Many people have worked upon this problem and no attempt will be made to review all of the results. We shall confine our discussion to a few papers closely related to our immediate problem. Wieland and Franke (6) have published an exhaustive paper upon the oxidation of organic acids by molecular oxygen in the presence of ferrous salts. In other papers (7, 8) they have used hydrogen peroxide instead of molecular oxygen as the oxidant. Their general conclusion is that the ferrous ion unites with the substrate thereby making the latter more susceptible to oxidation. Manchot (9) and others (10, 11) prefer to believe that the ferrous iron forms a peroxide and that this peroxide is the oxidant. One can recognize here the outlines of the two general theories of oxidation, that of peroxide formation and that of hydrogen activation.

If we apply to this problem the ideas stated at the beginning of this paper and others which follow from it we are led to the following considerations. If ferrous sulfate is added to tartaric acid, for example, some ferrous tartrate is formed, the amount formed depending upon the pH of the solution. We may assign to it the formula of Substance 1 in the accompanying scheme.

Substance 1   Substance 2   Substance 3

Substance 4   Substance 5   Substance 6

Substance 7   Substance 8   Substance 9   Substance 10

Substance 10

256
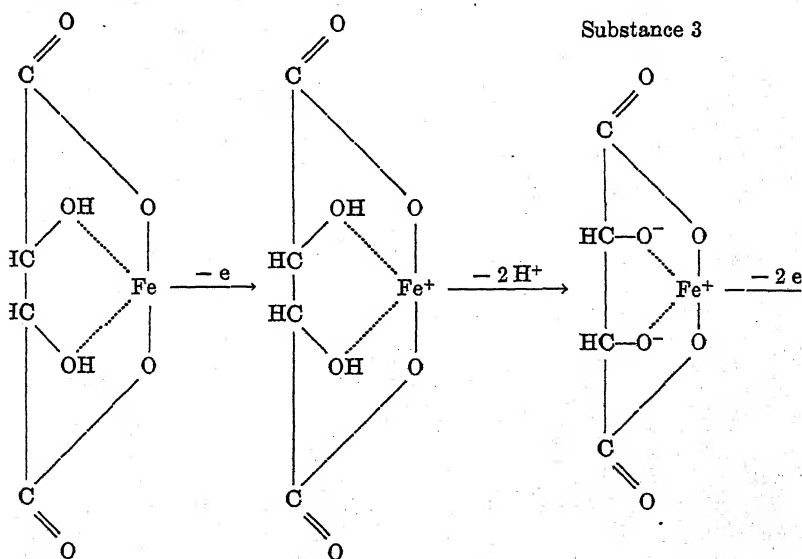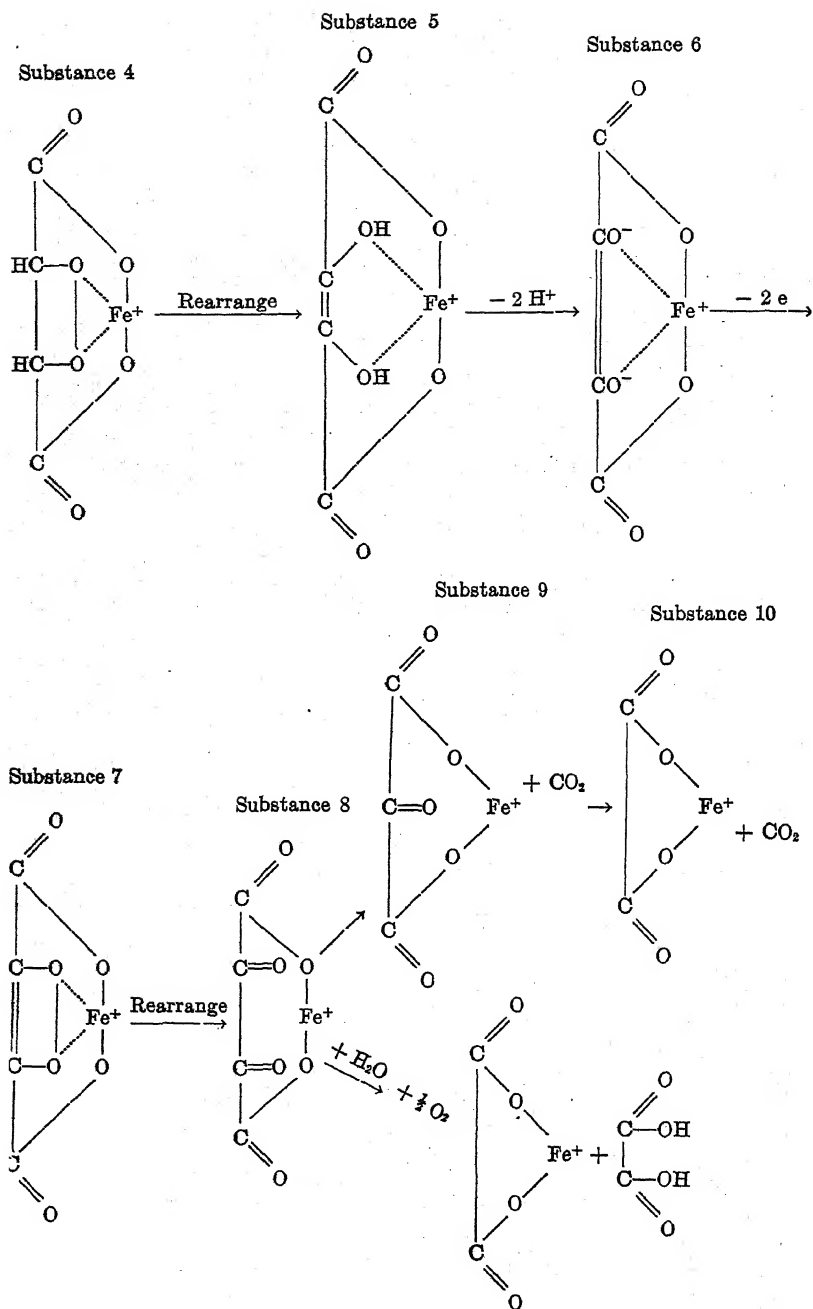
The dotted bond line is used here in the same sense that it was used by Smythe and Schmidt, to represent an attraction due to the residual negative charge on these oxygen atoms. For the calculation of this residual charge the reader is referred to the previous paper (1) or to the paper by Latimer and Porter (12). This iron is now electrically neutral ferrous iron so it can readily lose an electron to an acceptor such as molecular oxygen. The loss of this electron gives the iron a positive charge so it will draw in the electrons represented by the dotted lines. This makes the oxygen atoms involved more positive, in other words it makes the OH groups more acidic, and the hydrogens attached to them are to an appreciable extent forced off as hydrogen ions. In order to avoid confusion later we may point out that although we have ferric iron as soon as the electron separates from the ferrous iron our compound is not the usual ferric tartrate. The usual compound, formed when preformed ferric ion is added to tartaric acid, has the three iron valences attached to three carboxyl groups.

The electronic arrangement of the oxygens may now be represented as follows:
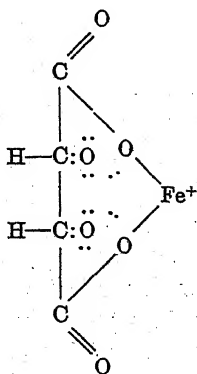
The iron is attracting a pair of electrons from each of the two oxygens.[1] If we consider these electrons to be in a dynamic condition it is easy to see that at some instant the iron will attract one pair to a greater extent than the other. This makes one

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[1] This may be regarded as what, in older terminology, was called a "zersplitterte" valence (13).

oxygen more positive than the other and the pair of electrons least attracted by the iron will be drawn toward the more positive oxygen, thus forming a bond between the two oxygens. The other pair of electrons passes to the iron and is immediately lost to an acceptor (molecular oxygen); or, stated in other words, the ferric iron is reduced and immediately reoxidized to the ferric state by the oxygen. Our compound is now represented as follows:

$$
\begin{array}{c}
O \\
\parallel \\
C \\
| \quad \diagdown \\
H{-}C{:}O \quad O \\
| \qquad \qquad Fe^{+} \\
H{-}C{:}O \quad O \\
| \quad \diagup \\
C \\
\diagdown \\
O
\end{array}
$$
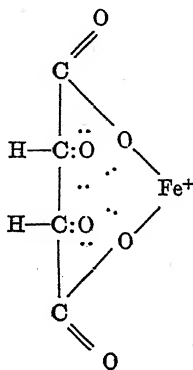
This ring involving the oxygen-oxygen linkage will rearrange to form Substance 5 in our scheme for this rearrangement is accompanied by a decrease in the sum of the products of the kernel charges of the atoms attached together (14). We are now in a position to repeat the process and lose two more hydrogens forming Substance 8. This will break down to form either 2 molecules of oxalate or it will lose 1 molecule of CO_2 at a time, first forming Substance 9. That the process actually occurs in a manner very similar to the above may be shown by the following facts. Fenton (15), who was one of the first to study this reaction obtained dihydroxymaleic acid (Substance 5 in the above scheme) in crystalline form from a mixture of tartaric acid and ferrous iron. He believed that this lost CO_2-forming Substance 9 in our scheme; but Wieland and Franke from a study of the O_2 consumed and CO_2 produced as well as by identification of the compounds themselves showed that the main reaction was to form Substance 8, which then gave Substances 9 and 10 in the ratio of 2:1. They found that Substance 9 slowly evolved CO_2. The fate of the ferric oxalate, Substance 10, will be discussed later.

If instead of adding ferrous iron to the tartrate we had added ferric iron the results would not be the same for here there is nothing to start the process; *i.e.*, there is no easily detachable electron analogous to the one possessed by ferrous iron. It does not follow, however, that ferric iron has no effect on the oxidizability of the tartrate. It should make it definitely more susceptible to oxidation for its secondary valences neutralize the residual negative charges on the oxygens of the alcoholic hydroxyl groups thereby making it easier for the hydrogens of these groups to separate.

Again the available evidence supports these predictions. Both Fenton (15) and Wieland and Franke (6) found that whereas ferrous ion markedly catalyzes the oxidation of tartaric acid, ferric ion is without measurable effect. However, when Wieland and Franke used a less inert oxidant, hydrogen peroxide, ferric ion showed a slow but definite catalytic effect. Similarly when they used a more readily oxidizable substrate, dihydroxymaleic acid, its oxidation by oxygen was catalyzed by ferric ion. This is to be expected for the alcoholic hydroxyl groups of this acid are more strongly acidic than those of tartaric acid, due to the adjacent double bond, and the secondary valences of the ferric iron enables them to ionize. In fact, a careful perusal of Wieland and Franke's entire paper has revealed nothing inconsistent with the considerations advanced here. The marked change, at higher pH values, in their curves for the oxidation of tartaric acid and dihydroxymaleic acid may require some explanation. I have repeated their experiments with tartaric acid, with solutions made up as they describe, but with only 1.6 cc. instead of the 20 cc. that they used, and I have obtained essentially the same results as Fig. 3 shows. The pH values recorded here are those reported by Wieland and Franke and refer to the solution after the ferrous sulfate has been added.

The striking thing about the above results is that the oxidation drops off very suddenly in the more alkaline solutions. If we remember that in these solutions we are dealing with a competition between tartrate and hydroxyl ions for the ferrous or ferric ions as the case may be, the explanation will become apparent. When the tartrate and ferrous sulfate are mixed, the higher the alkalinity of the solution the more ferrous hydroxide is formed. We know that ferrous hydroxide is rapidly oxidized and we know that the ferric
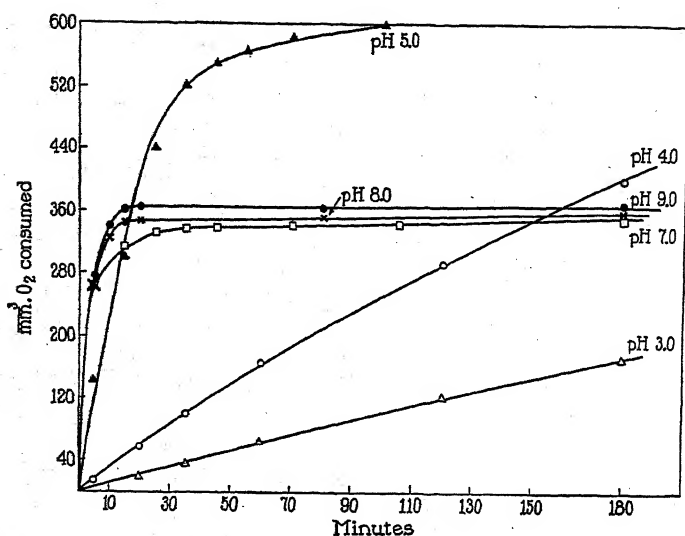
FIG. 3. Each flask contained 1.52 cc. of 1.0 M tartaric acid solution and 0.08 cc. of 0.5 M FeSO₄.   The O₂ equivalent of the iron present is 240 c.mm.
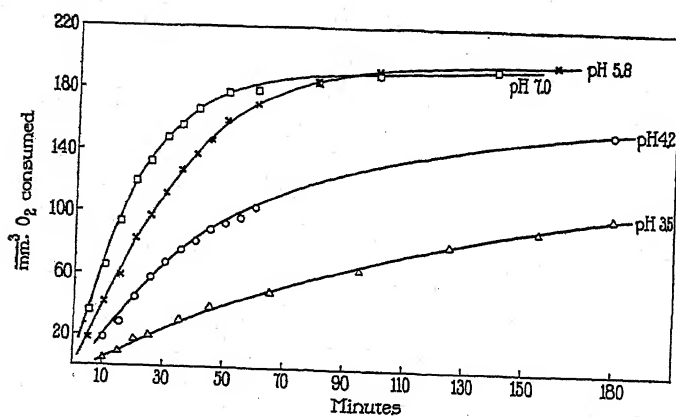

FIG. 4. Each flask contained 0.8 cc. of 0.2 M citric acid solution and 0.2 cc. of 0.1 M FeSO₄.   The O₂ equivalent of the iron present is 120 c.mm.
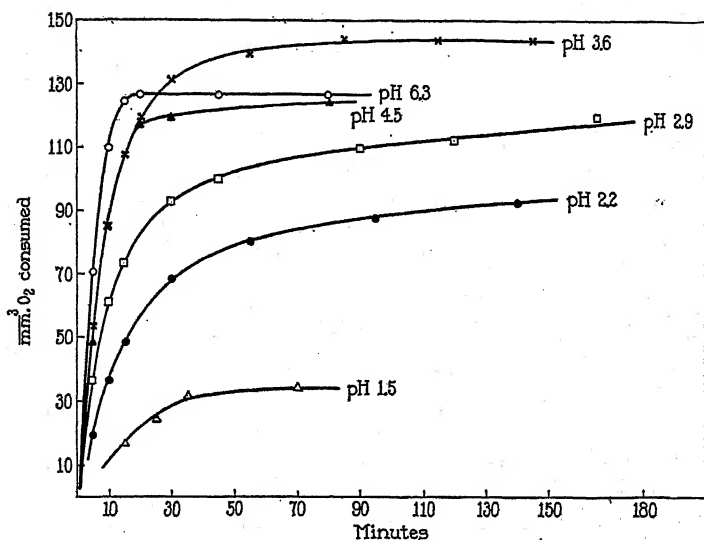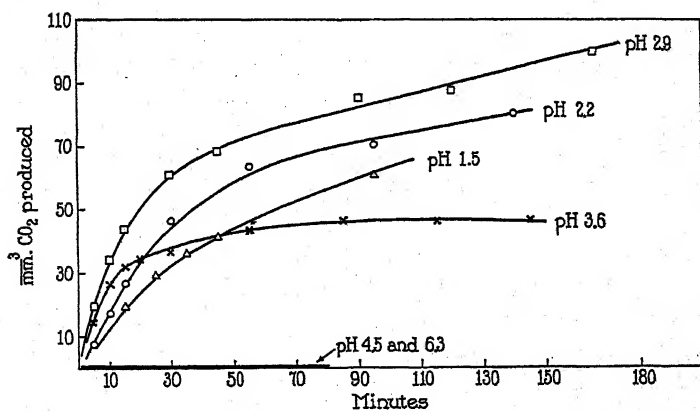
FIG. 5. Each flask contained 0.8 cc. of 0.2 M oxalic acid solution and 0.2 cc. of 0.1 M FeSO$_4$. The O$_2$ equivalent of the iron present is 120 c.mm.

FIG. 6. Each flask contained 0.8 cc. of 0.2 M oxalic acid solution and 0.2 cc. of 0.1 M FeSO$_4$.

iron so formed, even though it combines with tartrate, will have no catalytic action. Then if all the iron formed ferrous hydroxide the solution should take up just enough oxygen to oxidize the iron to the ferric condition and no more. Apparently Wieland and Franke obtained just this result at pH 9.0. The results shown in Fig. 3 do not show such a sharp end-point. Another factor that contributes to these results is the formation of ferric hydroxide from the ferric tartrate complex. We know that ferric hydroxide is a very insoluble substance and would tend to form to some extent at the more alkaline reactions.

Fig. 4 shows that the oxidation of citric acid at various pH values occurs just as our theory would predict. The pH values recorded refer to the citric acid solution before the addition of ferrous sulfate. It must not be imagined that the details given for the course of the oxidation with tartaric acid will apply without modification to the other acids.

We may now discuss the results with the simpler acid, oxalic, which may be an intermediate product in the oxidation of the above acids. The only oxidation product of this acid is carbon dioxide, so its determination is of interest to us here. It may readily be determined by running two vessels, one containing a compartment with alkali to absorb the CO_2 formed and the other without alkali. The difference in the pressure change recorded on the manometers of the two vessels represents the CO_2 absorbed. Fig. 5 shows the O_2 consumption when oxalic acid solutions of the pH values recorded are mixed with ferrous sulfate. Fig. 6 shows the accompanying CO_2 production. It may be seen that at the less acid reactions the oxygen consumption proceeds rapidly until an amount of oxygen approximately equivalent to the ferrous iron present is consumed, and then stops. A glance at Fig. 6 reveals that at these reactions there is no CO_2 production. The production of CO_2 reaches a maximum in the neighborhood of pH 2.9 and then drops off rapidly. It will be seen immediately that the CO_2 production occurs over just that range of acidity where mon-ovalent oxalate ion
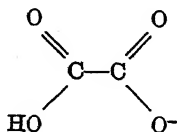
exists. This result agrees splendidly with our considerations. In ferrous oxalate
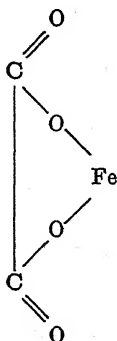
the iron should be readily oxidized, but there is no reason for the oxalate radical to be oxidized. This is an ordinary 5-membered ring and such rings are usually quite stable. This situation is not changed even if in excess of oxalate another molecule of oxalate is attracted by the residual valence of the iron. If, however, we have the monovalent oxalate ion present, the compound formed with ferrous ion is
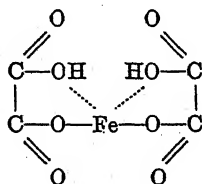
This will pass through a series of reactions entirely analogous to those discussed for tartrate. After losing two hydrogen ions and three electrons it will form
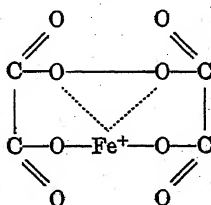
and there is no difficulty in seeing why this should lose CO_2. It should be pointed out that the oxygen uptake in excess of the iron equivalent in this case is very much slower than in the case of tartrate. One sample at pH 2.9 was run for 22 hours. During this time the CO_2 production was only 184 c.mm. The O_2 consumption, in excess of the iron equivalent, for the same period was 40 c.mm. It should be noted that oxalic acid produces 4 molecules of CO_2 for each molecule of O_2 consumed. The small oxygen consumption $\left(40 \text{ c.mm. instead of } \dfrac{184}{4} = 46 \text{ c.mm.}\right)$ may be partially due to the fact that not all the iron was in the ferric condition at the end.

Since, as we have seen, ferric oxalate is a stable substance, it follows, that, to the extent that this compound is formed in the oxidation of other organic compounds, the iron will be removed from the realm of active catalysis. In such cases the catalyst may be said to deteriorate during its action.

DISCUSSION

The important thing to be grasped from the above pages is that in a consideration of electron structure and residual charges on atoms may lie the explanation for that vague term "activation" of hydrogen. According to this concept the hydrogen separates from the molecule as hydrogen ion and the process of activation is simply a process of increasing the acid dissociation constant of the group involved. This is accomplished by the iron in the manner described.

Earlier in the paper we mentioned the fact that a number of workers consider that the ferrous iron functions, in such reactions as we have described, by forming an iron peroxide which then acts as the oxidant. The present paper throws no direct light upon this problem; however, we may point out that if molecular oxygen accepts the electron which ferrous iron gives up on being oxidized, it would seem quite necessary that there be a transient compound between them during the passage of the electron. This compound would be an iron peroxide. Such a compound would in no way change our scheme of oxidation and its existence is a matter outside the scope of this paper.

SUMMARY

It is shown that if ferrous iron is obtained in an unionized compound, it is readily oxidized to ferric iron by the oxygen of the air regardless of the acidity of the solution. An explanation in terms of electron structure is advanced.

The catalytic effect of ferrous and ferric iron in certain oxidations is discussed. An interpretation of the mechanism is proposed.

A suggestion is made concerning the meaning of the term activation of hydrogen.

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THE INEFFECTIVENESS OF MANGANESE IN NUTRITIONAL ANEMIA [1]

By W. E. KRAUSS

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The development of a technique (1, 2) whereby severe nutritional anemia can be induced in rats in the first generation has stimulated research in the field of hemoglobin regeneration. Several laboratories have taken up the problem and have published conflicting results. On one point there is universal agreement; namely, that the addition of iron and copper will cure nutritional anemia in rats on an exclusive whole milk diet or prevent the disease if added to the milk diet from the beginning. While Waddell, Steenbock, and Hart (3) have shown copper to be a specific factor in the prevention of nutritional anemia produced on a diet of whole milk and iron, a vast array of other elements has been presented by other workers as having a beneficial effect on hemoglobin regeneration.

Titus, Cave, and Hughes (4) found manganese very effective and as a result of their work concluded: "Manganese added to a milk-iron diet seems to give almost, if not quite, as good results in the building of hemoglobin as does copper added in the same way." Myers and Beard (5) also found manganese, as well as a number of other elements, to be effective in bringing about hemoglobin regeneration in anemic rats. That something besides copper was responsible for the results obtained by other workers was the contention made by Drabkin and Waggoner (6) who, by feeding a synthetic copper-free ration, succeeded in curing rats suffering from anemia induced by a whole milk diet. It has, however, been shown by Elvehjem and Hart (7) that the so called copper-free synthetic ration of Drabkin and Waggoner was not copper-free.

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The results obtained by Titus, Cave, and Hughes (4) and by Myers and Beard (5), together with the contrary results obtained by Waddell, Steenbock, and Hart (3) when they employed the same manganese salt used by the Kansas workers, made it essential that further work be done on the rôle of manganese in hemoglobin regeneration. At the suggestion of Dr. E. B. Hart of the University of Wisconsin, and with the approval of Dr. J. S. Hughes of the Kansas Agricultural College, it was decided to include this phase of the problem in the program on nutritional anemia being carried out in the nutrition laboratory of the Dairy Department of the Ohio Experiment Station.

<div align="center">EXPERIMENTAL</div>

A sample of copper sulfate and one of manganese chloride, together with a solution of ferric chloride, were obtained from Dr. Hart. Solutions of manganous chloride and iron chloride were received from Dr. Hughes. These samples were part of the supply used previously in the respective laboratories of these workers.

The copper sulfate and manganese chloride salts from the Wisconsin laboratory were made up to contain 0.05 mg. of copper and 0.1 mg. of manganese per cc., respectively. The Wisconsin iron solution, as received, contained 5 mg. of iron per cc. This was diluted for feeding so as to contain 0.5 mg. of iron per cc. As received, the Kansas solution of iron chloride contained 15 mg. of iron per cc., and the manganese chloride solution, 5 mg. of manganese per cc. These were diluted so as to contain 0.5 mg. of iron and 0.1 mg. of manganese per cc., respectively. As no copper salt or solution was submitted by Dr. Hughes, one containing 0.05 mg. of copper per cc. was prepared from a supply of copper sulfate previously used in our own laboratory. All dilutions were made with water redistilled from glass. Qualitative tests revealed no manganese in the copper solutions and no copper in the manganese solutions.

Albino rats were used in this work. The technique of feeding, handling, and weighing the rats and of making hemoglobin determinations has been previously described (2, 8). For almost the entire period covered, the milk used was taken from the combined production of three Holstein cows kept and fed under normal winter

feeding conditions. For a short time toward the close of the trials milk from Holstein cows having access to pasture was used.

Since the chief point under consideration in this problem was the effectiveness of a combination of iron and manganese in nutritional anemia of the rat, twelve animals received this combination after having developed severe anemia, seven receiving additions of the Kansas salts and five of the Wisconsin salts. As it was desirable to know the effectiveness of each element alone and of various combinations other than manganese and iron, other groups of rats were so fed as to determine these points. In all, the following groupings were made, fewer animals being used in those groups on which data had been previously obtained.

Group   I. Milk exclusively.
"   II. " + 0.5 mg. Fe (K.).
"   III. " + 0.5 "   "   (W.).
"   IV. " + 0.05 "   Cu (O.).
"   V. " + 0.05 "   "   (W.).
"   VI. " + 0.10 "   Mn (K.).
"   VII. " + 0.10 "   "   (W.).
"   VIII. " + 0.5 "   Fe (K.) and 0.1 mg. Mn (K.).
"   IX. " + 0.5 "   "   (W.) " 0.1   "   " (W.).
"   X. " + 0.1 "   Mn (K.) " 0.05 "   Cu (O.).
"   XI. " + 0.1 "   "   (W.) " 0.05 "   " (W.).
"   XII. " + 0.5 "   Fe (K.) " 0.05 "   " (O.).
"   XIII. " + 0.5 "   "   (W.) " 0.05 "   " (W.).

K. was material from the laboratory of Dr. J. S. Hughes, Manhattan, Kansas; W. from the laboratory of Dr. E. B. Hart, Madison, Wisconsin; O. from the laboratory of Dr. W. E. Krauss, Wooster, Ohio. The writer is indebted to Dr. Hughes and Dr. Hart for furnishing these materials and for their helpful suggestions.

In addition, three rats were fed iron and manganese from the beginning, furnishing limited prophylactic data which, however, was entirely substantiated by previous work.

The additions were made by pipetting 1.0 cc. of each of the designated solutions into a small amount of milk for each rat at the afternoon feeding. This insured quantitative consumption. The levels of feeding the iron and copper were those previously found effective by Hart, Steenbock, Waddell, and Elvehjem (9); the level of manganese feeding was that used by Titus, Cave, and Hughes (4).
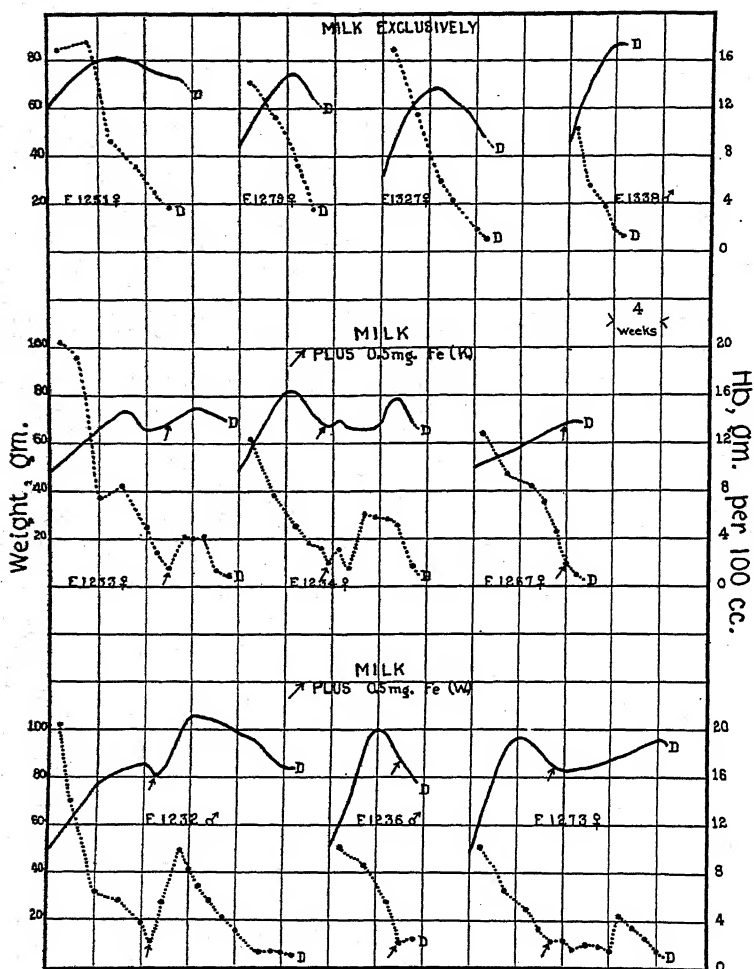
CHART I.   The effects of an exclusive milk diet and of the addition of Fe (K.) and Fe (W.) are shown by these curves.   Both iron salts brought about a temporary stimulation in hemoglobin production.

In this and all succeeding charts, the solid lines represent weight and the broken lines hemoglobin.   K. represents Kansas; W., Wisconsin; O., Ohio; the arrow indicates the point at which the supplement was added; and D indicates dead.
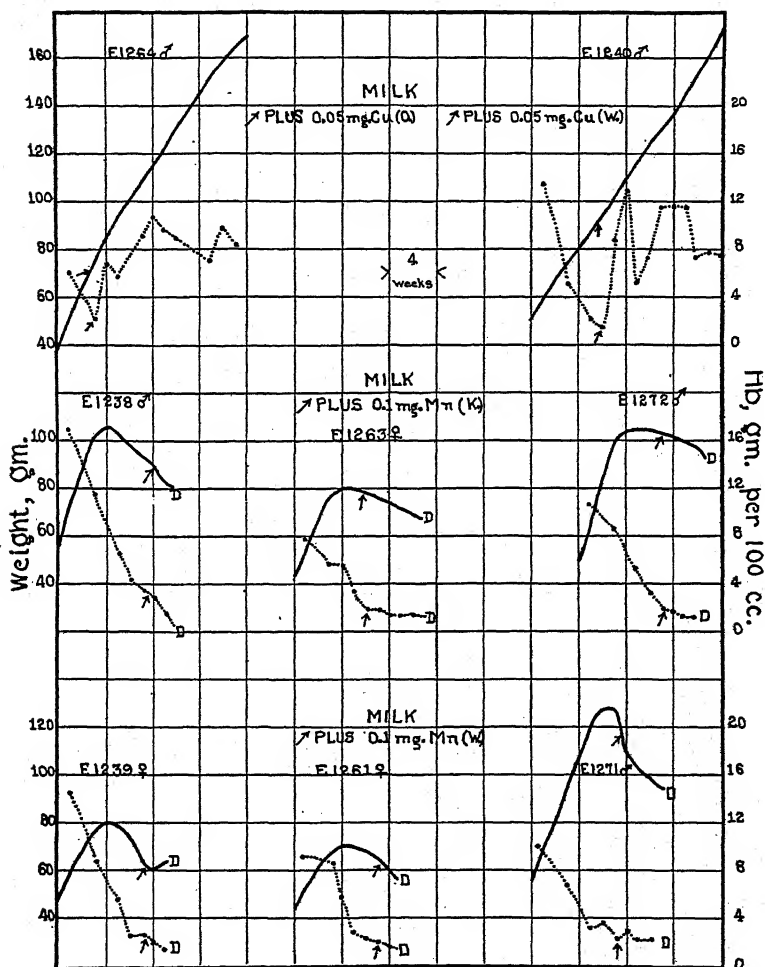
CHART II.   Some response was obtained by the addition of copper alone. The behavior of Rats E 1264 and E 1240 was similar to that of a number of others observed in this laboratory.   The addition of manganese from either source did not prevent a continued drop in hemoglobin.

Results

The results are shown graphically in the charts. Curves for only four control animals are shown (Chart I), but during the course of
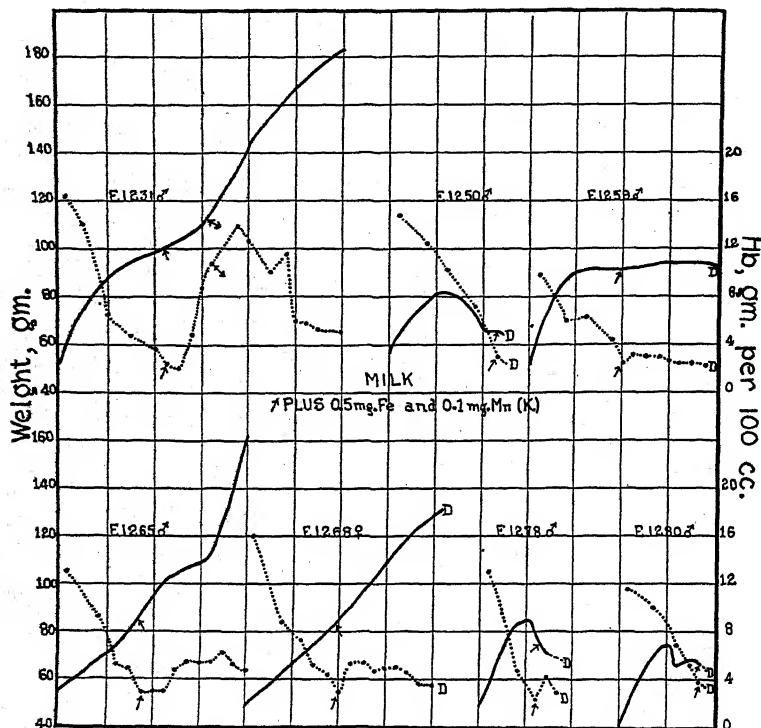
CHART III. The ineffectiveness of Fe (K.) and Mn (K.) combined is shown here. The slight response upon the addition of Fe and Mn was no greater than that obtained by the addition of Fe alone (Chart I) and was probably due only to the iron. The behavior of Rat E 1231 at the point indicated by an arrow may be attributed to gnawing on a piece of copper wire which was attached to the cage. Removal of the wire at the point indicated by a double arrow resulted in the development of anemia after the copper reserve had been used up.

this work many more rats developed nutritional anemia on the same milk. The addition of iron from either source, in four cases out of six, slightly raised the hemoglobin temporarily and prolonged life for several weeks (Chart I). Eventually, however, the

rats died.   When copper was added, the hemoglobin was raised considerably, growth was continued, and life was indefinitely pro-
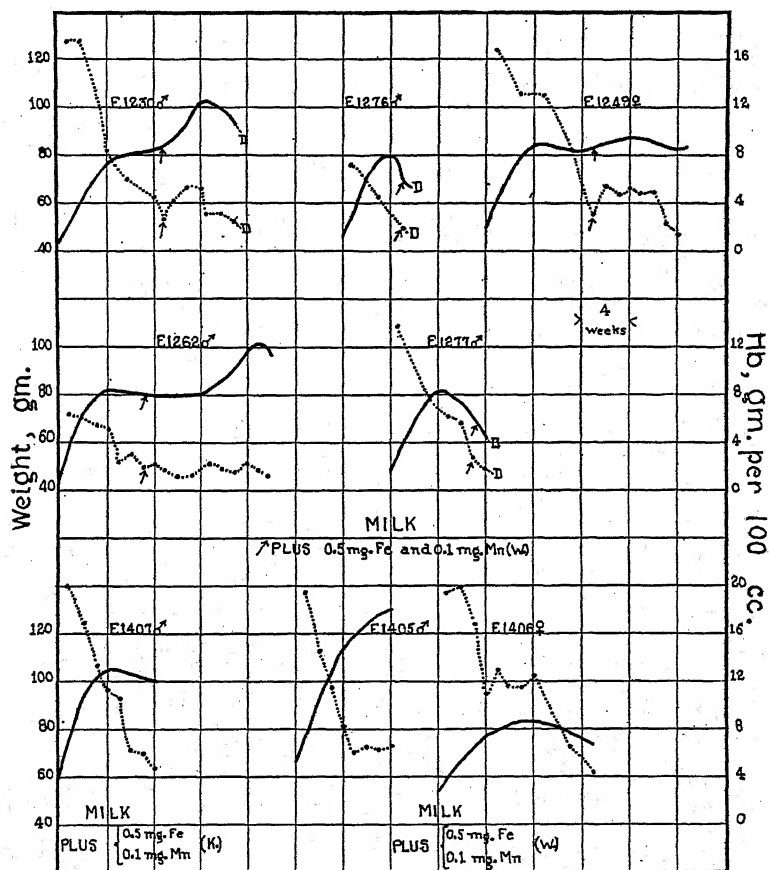

CHART IV. The ineffectiveness of Fe (W.) and Mn (W.) combined, as a curative, and of salts from both sources as prophylactics, is emphasized here.

longed (Chart II).   These effects of the addition of iron and copper, respectively, have been previously observed in this laboratory.

The addition of manganese from either source did not stimulate hemoglobin regeneration, nor did it prevent the decline in hemo-

globin and ensuing death (Chart II).   When iron and manganese were added simultaneously the response was similar to that obtained by the addition of iron alone and was probably due to the iron only (Charts III and IV).   Attention is called to Rat E 1231♂ which received, after its hemoglobin had fallen to 2.0 gm. per 100 cc. of blood, the regular additions of iron and manganese.   After a
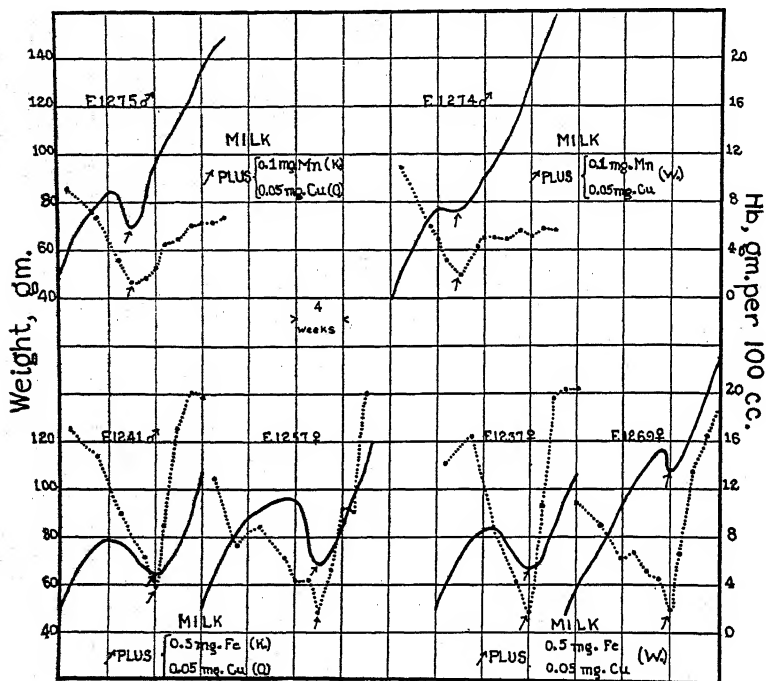

CHART V.   The combination of Mn and Cu was no more effective than Cu alone, as shown here.   Fe and Cu, fed simultaneously, resulted in phenomenal response.

further drop in hemoglobin during the following week sudden improvement in the animal was observed.   This continued for 3 weeks until it was discovered that attached to the cage was a piece of copper wire which had obviously been gnawed by the rat. This wire was removed and in the course of 2 weeks the rat was developing anemia.   That the rat developed anemia in the first

place was probably due to the fact that it had not discovered the wire.

Supplements to deficient diets are usually more effective as prophylactics than as curatives. However, when iron and manganese were added simultaneously from the time the rats were placed on milk, anemia developed with the same rapidity as when the usual milk-iron diet was fed. In addition to the data represented by the curves for Rats E 1405, E 1406, and E 1407 (Chart IV), it had been found in this laboratory previously that the addition of manganese sulfate to a milk diet did not prevent anemia, nor did the addition of the Daniels and Hutton salt mixture,[1] which contains manganese (10).

The combination of copper and manganese produced the same result as when copper alone was added (Chart V). On the other hand, when copper and iron were fed simultaneously, the amount of copper being only half the amount of manganese fed, the response was immediate and phenomenal. The remarkable effect of copper is emphasized by the reaction of Rat E 1241 (Chart V). When the hemoglobin level of this animal had dropped to 4.0 gm. per 100 cc. of blood the animal was not only suffering from severe anemia, but from an acute intestinal infection. This animal could well have been discarded as its death was imminent, but copper and iron were administered and in the course of 3 weeks the rat was apparently a normal individual.

DISCUSSION

A study of the graphs reveals that no difference in response resulted when salts prepared in different laboratories were fed simultaneously to rats of the same litter. The fact that neither the Wisconsin workers nor ourselves could demonstrate any beneficial effect through the use of iron and manganese salts from the same source as those used by Titus, Cave, and Hughes would indicate that some contamination existed in the materials in the Kansas laboratory. This theory is strengthened by the inability of Hughes[2] to repeat the results first obtained.

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[1] AlK $(SO_4)_2 \cdot 12H_2O$, NaF, Na_2SIO_3, and $MnSO_4 \cdot 4H_2O$ were fed so that each animal received 1.5 mg. of each salt daily. 1 drop of a 2 per cent solution of sodium iodide and 3 drops of a saturated solution of iron citrate were added to each 200 cc. of milk.

[2] Personal communication from Dr. Hughes.

The ease with which a contamination may creep into this type of work is demonstrated by our experience with Rat E 1231, reported in this experiment (Chart III), and with a few others.   The source of contamination may not always be detectable.   In one instance we found it impossible to make a group of four rats, housed in one cage, anemic on an exclusive milk diet.   A close examination of the cage and all equipment revealed nothing; however, when the rats were removed to another cage they promptly developed anemia.   The desirability of using glass equipment throughout is apparent as this would preclude the possibility of metallic contamination.

The argument may be advanced that the milk used in our work contained less manganese than that used by Titus, Cave, and Hughes.   Analysis of a preserved composite sample of the milk used in this trial was made in the laboratory of Dr. Hart.   It was found to contain 0.034 mg. of manganese per liter,[3] as compared to 0.020 mg. per liter in milk sent to the Wisconsin laboratory by Dr. Hughes.   It is not likely that a deficiency of manganese in the milk used by us could be responsible for the lack of response when 0.1 mg. of manganese was added daily.   Further refutation of this argument is advanced by the work of Waddell, Steenbock, and Hart (3) who fed as high as 5.0 mg. of manganese per rat per day without effect.

The phenomenal responses we have obtained by the addition of copper under extremely adverse conditions has emphasized the rôle played by this element as a specific factor for hemoglobin regeneration in rats suffering from nutritional anemia.

Further evidence of the specificity of copper and the inadequacy of manganese was offered by Lewis, Weichselbamm, and McGhee (11) who found electrolytically prepared iron and copper effective while manganese, prepared from manganese sulfate (c. p. Baker's) and fed with the electrolytically prepared iron, produced no response.

Myers and Beard (5) and Beard and Myers (12) reported favorable responses through the use of Fe, Cu, Ni, Ge, As, Mn, Co, Ti, Zn, Rb, V, Cr, Se, or Hg.   Recent work of Mitchell and Miller (13) also indicates that a number of inorganic elements may be concerned.   While no criticism of the technique of these workers is

[3] Analysis of the fresh milk used in our work gave somewhat higher results, ranging from 0.042 to 0.059 mg. per liter.

intended or can be justly made without further investigation, it is difficult to conceive how so many substances can be concerned in the performance of so specific a function as hemoglobin regeneration.

SUMMARY

Data are presented showing the effect of salts of iron, copper, and manganese furnished by different laboratories on hemoglobin regeneration in rats suffering from nutritional anemia induced by an exclusive liquid whole milk diet.

Manganese, when added alone (0.1 mg.) or in combination with iron (0.5 mg.), did not bring about improvement in rats suffering from nutritional anemia. The conclusion reached by Titus, Cave, and Hughes that "Manganese added to a milk-iron diet seems to give almost, if not quite, as good results in the building of hemoglobin as does copper added in the same way" was not upheld.

The conflicting results obtained in the laboratories from which the salts were obtained as to the response of rats suffering from nutritional anemia were probably not due to impurities in the salts used but possibly to slight differences in the technique of housing and feeding the experimental animals.

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FURTHER EVIDENCE OF THE COMPLEX NATURE OF VITAMIN B

II. EVIDENCE THAT A THIRD FACTOR EXISTS*

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In previous papers (1, 2) it was shown that at least three factors exist in the vitamin B complex. Other investigators (3, 4) have also presented evidence that a third and possibly a fourth factor exists. Much of the evidence presented in favor of the existence of the third factor has been based upon the effect of heat, in the presence of different pH values, upon yeast. In this paper additional evidence is presented to show that a third factor exists in this exceedingly complex vitamin B. A large quantity of the two fractions has been prepared and studied. The data in this paper were obtained by feeding the prepared fractions from yeast and rice polishings alone and with natural products. The methods of preparing the fractions are given below.

Method

From Yeast—Dry powdered yeast was suspended in 0.1 per cent acetic acid in the proportion of 1 kilo of yeast to 4 liters of acidulated water and allowed to stand 24 hours. The supernatant liquid was then siphoned off and 2000 cc. of 0.1 per cent acetic acid were added and well shaken. It was allowed to stand another 24 hours when the supernatant liquid was siphoned off again. This was repeated for a total of fourteen times. The resulting residue was dried before an electric fan, at room tem-

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perature. The extract was concentrated to about 5 liters before an electric fan at room temperature, and its pH value adjusted to about 4.5. It was then supercentrifuged to remove the suspended solids. The solids were discarded while the clarified extract was treated twice with fullers' earth, in the proportion of 50 gm. per 1000 cc. of the extract. After shaking occasionally for several hours it was filtered on a Buchner funnel. The earth was washed several times with 0.1 per cent acetic acid. The filtrate was concentrated before an electric fan until 1 cc. was equivalent to 1 gm. of the original yeast. In this paper it will be designated as the B or non-adsorbable fraction.

Another fraction of the vitamin B complex was removed from the moist fullers' earth by means of a 2.5 per cent solution of barium hydroxide. The liquid was separated from the earth by filtering and washing with suction on a Buchner funnel. This extraction was repeated five or six times, and washed thoroughly with water. The filtrate was removed frequently from the suction flask and acidified with H_2SO_4. The filtrates were combined and the $BaSO_4$ was removed by centrifuging. The liquid was then concentrated before an electric fan until 1 cc. was equivalent to 1 gm. of the original yeast. This fraction is designated as the A fraction, and contains the antineuritic vitamin (vitamin B or F). The fullers' earth residue was dried at room temperature.

From Rice Polishings—2 kilos of rice polishings were suspended in 6 liters of 0.1 per cent acetic acid, and allowed to stand 24 hours, when the supernatant liquid was siphoned off. About 4 liters of 0.1 per cent acetic acid were added to the solids, stirred, and allowed to stand another 24 hours, when the supernatant liquid was siphoned off. This procedure was repeated four times. The extracts were combined and evaporated at room temperature before an electric fan to an approximate volume of twice the original weight of the polishings. The extract was then treated with a solution of lead acetate, as suggested by Rosedale (5). The antineuritic fraction (non-precipitated fraction) prepared by this method had a potency equivalent to that prepared from yeast. It is also designated as vitamin B or vitamin F.

Rats 24 days of age and weighing from 50 to 60 gm. were used as the experimental animals. They were kept individually in screen bottom cages. The basal vitamin B-free diet consisted of casein 18, starch 64, Salt Mixture 185[1] 4, Crisco 10, agar-agar 2, and cod liver oil 2. The vitamin preparations were fed daily and separately. In a previous communication (6) it was shown that a similar synthetic diet containing 25 per cent of wheat, plus 0.4 gm. of autoclaved yeast, daily, supplied the necessary vitamins in sufficient quantity for normal growth and prevention of pellagra. This raised the question, "Does autoclaved yeast contain any other vitamin or vitamins, aside from vitamin G (antipellagra or P-P factor)?" All attempts to produce growth that anywhere approaches normal with vitamin B (adsorbable fraction) and the non-adsorbable fraction (treated with fullers' earth) from yeast extract have failed. As a rule, rats on a diet of 25 per cent of wheat and the non-adsorbable fraction (fraction B) from yeast extract failed to grow, but when the non-adsorbable fraction was fed in addition to the yeast residue, growth was excellent. It is evident from this that the non-adsorbable fraction is not equivalent to autoclaved yeast, but that the non-adsorbable fraction with the yeast residue, or the yeast residue alone, is equivalent to autoclaved yeast in preventing pellagra (Chart 1). This suggests that in the preparation of an acidulated water extract of yeast something essential remains adsorbed on the protein of the residue, and which is very difficult to remove, although, as shown later, some of it dissolves when yeast is extracted with 0.1 per cent acetic acid. This also suggsts that there are at least two fractions, a growth fraction and the antipellagra fraction, other than the antineuritic fraction, present in yeast, and two fractions in autoclaved yeast. Chart 1 also shows the growth curves of rats receiving in addition to the basal diet 0.4 gm. of autoclaved yeast plus 1 cc. of the antineuritic fraction, and 2.0 gm. of autoclaved wheat plus 1 cc. of the antineuritic fraction, respectively.

[1] McCollum's Salt Mixture 185 (McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918)).

This may indicate that autoclaved yeast contains some factor or factors that are absent, or practically so, in wheat.
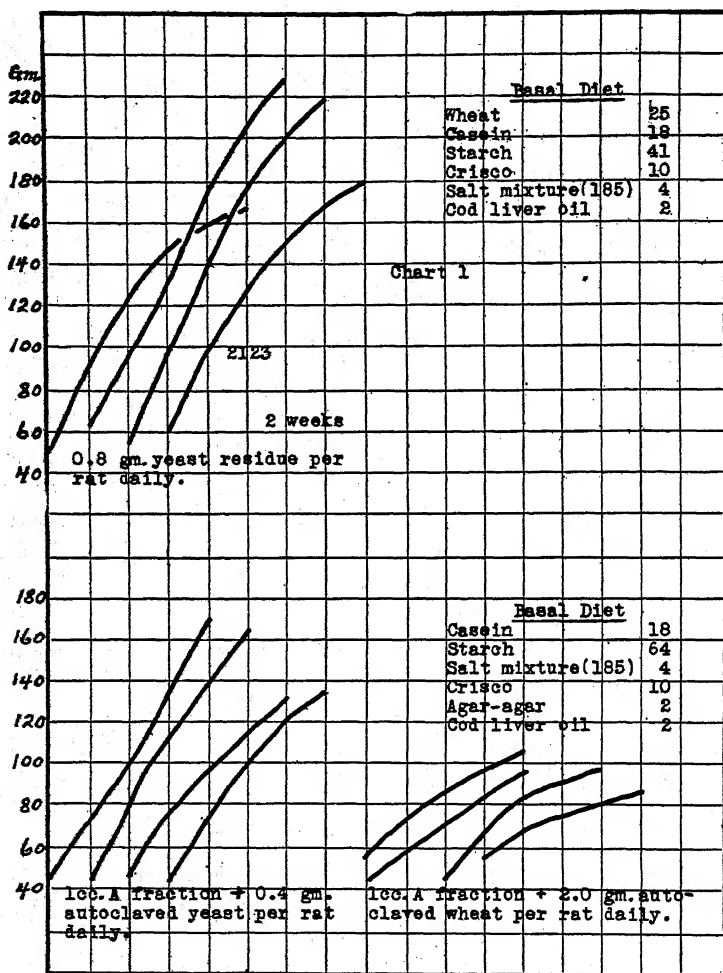

CHART 1

An extract of yeast of proper concentration produces excellent growth in rats, placed on a diet adequate in every respect except the vitamin B complex, while the adsorbable and non-adsorbable

fractions (treated with fullers' earth) from the yeast extract
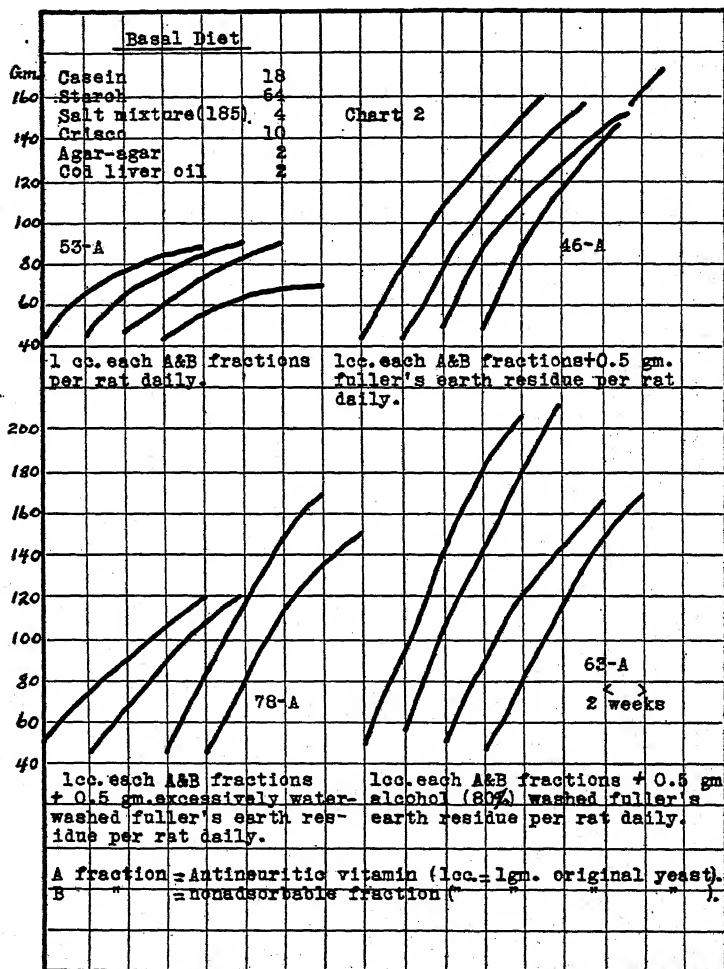failed to produce growth, as was shown in a previous paper (2)

CHART 2

as well as in Chart 2. It was concluded that the fullers' earth
had removed something from the extract that was essential for
the growth of the animal (Chart 2). Excessive washing of the

fullers' earth with water and 80 per cent alcohol, respectively, has failed materially to remove the factor.   This is shown by the growth curves in Chart 2, and is in agreement with the data presented by .Narayanan and Drummond (7).   Other reagents
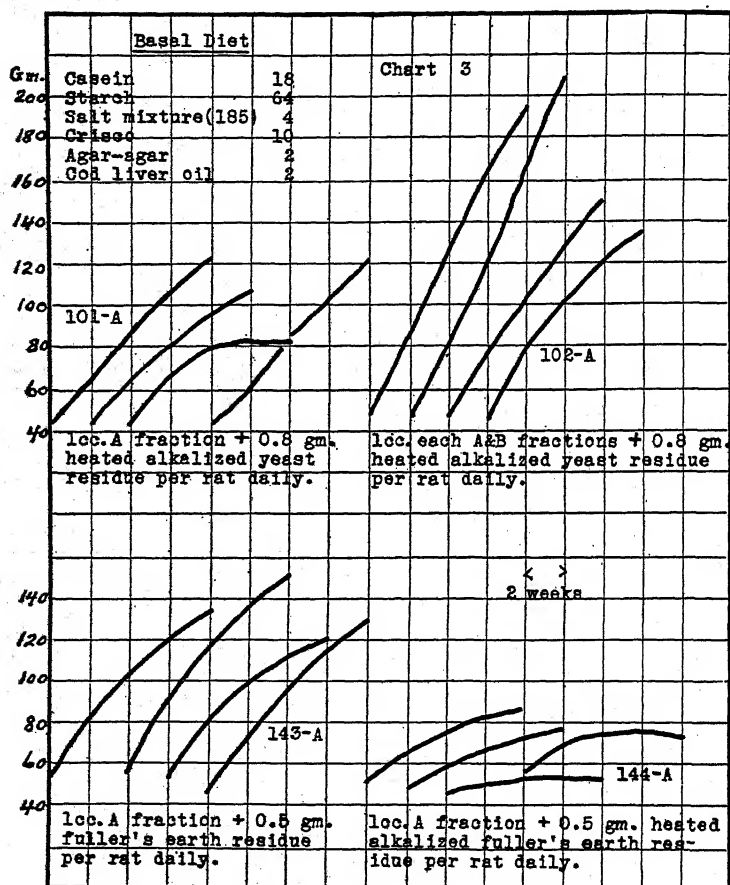

Chart 3

for removing this fraction from the fullers' earth residue are being tried.   The fact that the animal has no trouble in removing this fraction indicates the complexity of the process taking place in the animal's digestive tract.

In a previous publication (2) it was shown that the factor

which was present in the yeast residue was thermostable. Further investigation has confirmed this and also shown that there are two factors present in yeast and fullers' earth residues, respectively, one of which is thermostable in either an acid or alkaline medium, while the other is thermolabile in an alkaline medium only. It is believed that the thermolabile factor is the B or non-adsorbable fraction which remains adsorbed as an impurity. For that matter both factors are evidently impurities but one is more easily removed than the other. This

Chart 4

1cc. each A&B fractions + 0.8 gm. autoclaved alkalized yeast residue per rat daily.

1cc. A fraction + 1cc. autocl. alkalized B fraction + 0.8 gm. autocl. alkalized yeast residue per rat daily.
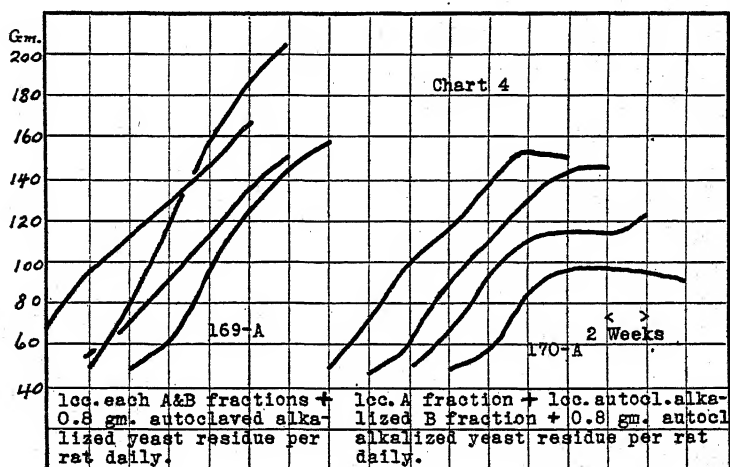
169-A

170-A

2 Weeks

CHART 4

conclusion is supported by the growth curves presented in Charts 3 and 4. Further confirmation will be shown later.

In order further to confirm or reject the hypothesis that an essential factor exists in the yeast residue, and to attempt to elucidate the fact that the yeast extract when administered to animals in proper concentration, or amounts, causes them to grow normally, it was thought that the problem might be attacked by centrifuging the extract of yeast. The thought was that an extract of yeast contained suspended matter which had growth-promoting qualities. For this purpose an extract of yeast was divided into two portions, one of which was run through a supercentrifuge, operating at 36,000 to 40,000 revolutions per minute. The other

portion was allowed to stand 12 hours, or longer, and the upper
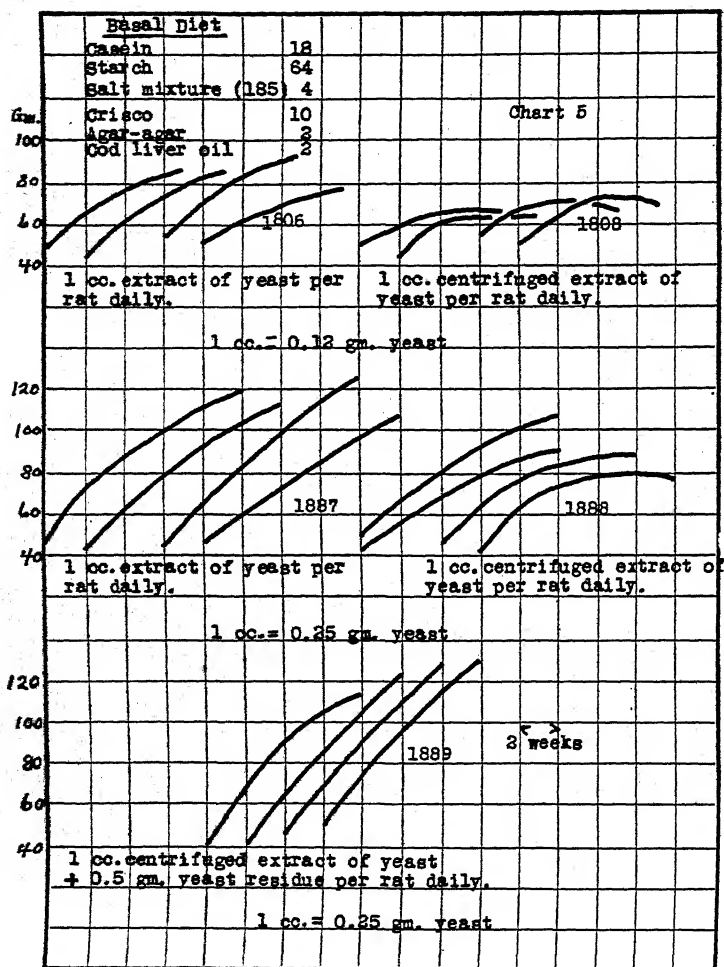layer was decanted off. The two portions were fed at two con-

CHART 5

centrations to rats as the sole source of the vitamin B complex,
with results as shown in Chart 5. The effect of adding the yeast
residue to the centrifuged portion on the growth of rats is also

shown in Chart 5. The results seem to indicate that the yeast extract contains some substance in suspension that stimulates the
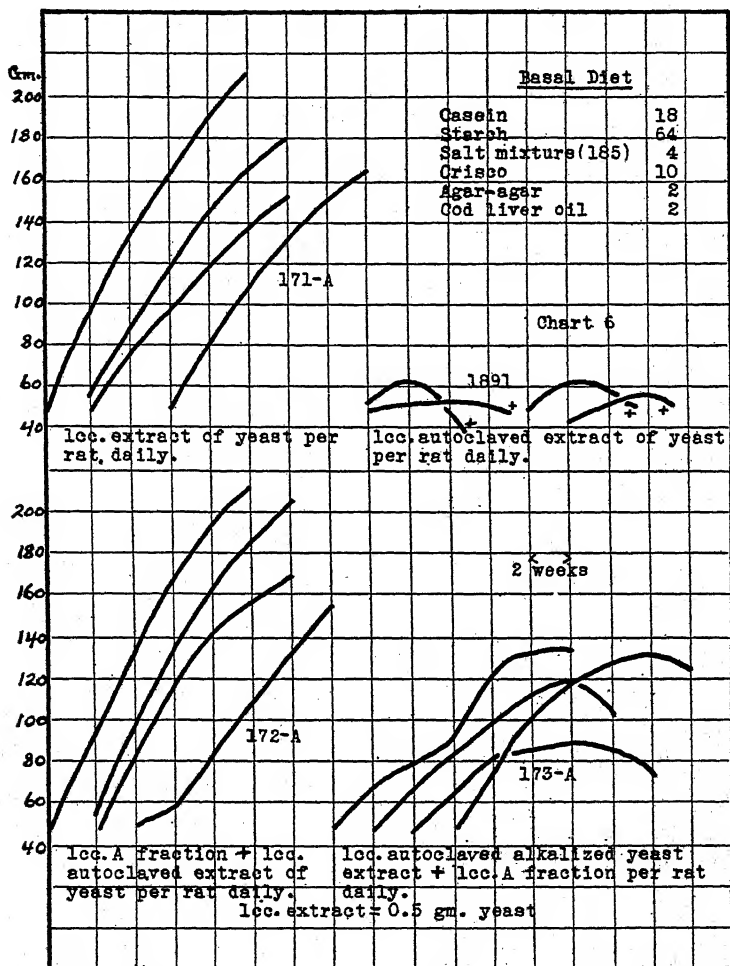

CHART 6

growth of rats and which can be partially removed by super-centrifuging.

As is well known, yeast extract has been found to be an ade-

quate source of the vitamin B complex, for rats grow normally when the extract and a diet adequate in every respect, except the complex vitamin B, are fed.   This gives assurance that the three factors, and possibly others of this complex, are present in the extract.   It is true that one factor is heat-labile under one condition, and another one is heat-stable under all conditions, etc.; then one should be able to differentiate them by autoclaving different portions of the extract under different pH conditions. An extract of yeast was prepared from dry whole yeast, by the method previously described.   This extract was divided into three portions.   One was fed as prepared, the second portion was
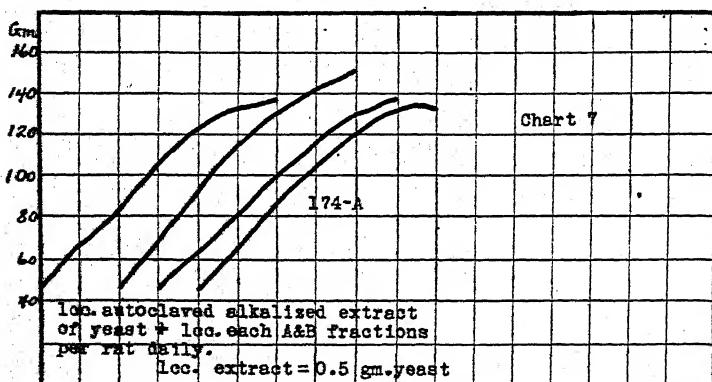

CHART 7

autoclaved as prepared (pH 5.1), and the third portion was made alkaline (pH 9.4), autoclaved for 5 hours at 15 pounds pressure, and then made slightly acid.   The autoclaved acid extract was fed alone and with the addition of vitamin B (antineuritic vitamin). The autoclaved alkalized extract was fed with the addition of the vitamin B alone, and with the vitamin B and the non-adsorbable fullers' earth fraction, respectively.   It is evident from the results shown in Charts 6 and 7 that one fraction, vitamin B, of the extract is heat-labile in an acid medium, another one (non-adsorbable fraction) is heat-labile in an alkaline medium, and the third fraction (antipellagric or vitamin G) is thermostable under all of the conditions tried in this laboratory.   It will be noticed

that when the fractions which are supposed to be destroyed in the extract, under the above conditions, are added, a response in growth is observed. In some cases the response was not as great

Gm.

Basal diet   Chart 8

Casein	18
Starch	64
Salt mixture (185)	4
Crisco	10
Agar-agar	2
Cod liver oil	2

180
160
140
120
100
80
60
40

0.8 gm. yeast residue.   0.5 gm. fuller's earth residue.

120
100
80
60
40

2 weeks

0.8 gm. autoclaved alk-   0.8 gm. autoclaved alkalized yeast
alized yeast residue.   residue + 1cc. B fraction.

60
40
100
80
60

2cc. B fraction.

+Death

4cc. B fraction.

40

Pellagrous symtoms were induced by feeding the basal diet
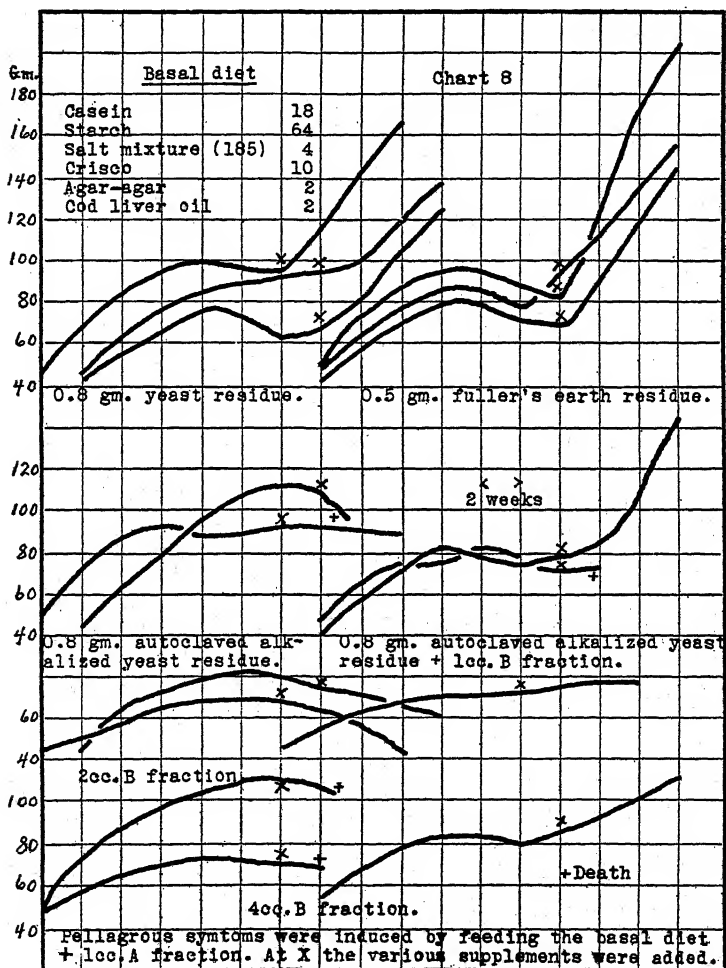+ 1cc. A fraction. At X the various supplements were added.

CHART 8

as the theory would predict, but this may be due to the incomplete destruction of that particular fraction in the original extract. Three of the four rats receiving the autoclaved alkalized yeast ex-

tract and vitamin B developed pellagrous symptoms. This agrees in substance with Reader's work (4), and is not in agreement with the results obtained by Chick and Roscoe (8). Further work is in progress to test the validity of the above interpretations.

All the criteria thus far had been obtained by growth observations, or in other words preventive measures had been used. It was thought pertinent to attack the problem, as far as the pellagra conditions were concerned, from a curative standpoint. However, growth and pellagra are so intimately associated that it is very difficult to dissociate them. Experimental pellagra was induced by feeding the animals the synthetic vitamin B complex-free diet, and the vitamin B (antineuritic fraction). All the rats developed symptoms indicative of pellagra in 10 to 12 weeks. At the end of the 12th week the rats were placed in individual cages and fed the different supplements indicated in Chart 8. From the growth curves shown in Chart 8 it is evident that the fullers' earth and yeast residues respectively contain some substance which cures pellagra. This was confirmed by macroscopic examination of each rat. It was the belief that the fullers' earth and yeast residues still retained some of the non-adsorbable fraction which is one factor in the prevention of pellagra. To test this out, and to confirm or reject a previous hypothesis, the yeast residue was made alkaline (pH 8.8) and then autoclaved for 2 hours at 15 pounds pressure. When this was fed as an additional supplement to the pellagra-producing diet, pellagra was not cured. However, when the non-adsorbable fraction (B fraction) was fed in addition to autoclaved alkalized yeast residue, pellagra was cured, indicating that the alkali heat-labile and thermostable substances are necessary to cure pellagra. The non-adsorbable fraction (B fraction) did not cure pellagra (Chart 8). The results shown in Chart 8 also indicate that the antipellagra, or antidermatitis factor is not the non-adsorbable fraction of yeast extract, as previously reported, (2) but that it is probably an adsorbable fraction, different from the antineuritic fraction. The third factor is present in the non-adsorbable fraction, and it is this factor, along with vitamins B and G, which is essential for growth, as well as the prevention and cure of pellagra. All three factors are apparently necessary for normal growth and well being of the rat.

SUMMARY AND CONCLUSION

New evidence is presented to show that a third.factor exists in the vitamin B complex. This factor is distinct from the antineuritic and the antipellagric factors.

All three factors seem to be necessary for the growth of the rat.

The antineuritic factor (vitamin B) is heat-labile in an alkaline and acid medium; the antipellagric factor (vitamin G) is thermostable under all conditions tried, while the evidence presented seems to show that the third factor is thermolabile in an alkaline medium (pH 9) when autoclaved for 5 hours at 15 pounds pressure.

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NOTE ON THE PREPARATION OF HYDROXYPROLINE

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During the preparation of large quantities of proline in this laboratory a simple method for the isolation of hydroxyproline, as a by-product, has been developed.

Town's (1) procedure, which depends upon the separation of the hydrolysis products of protein by means of the solubilities of their copper salts, was used for the isolation of proline. By this method the amino acids are divided into the following fractions; (Fraction 1) copper salts insoluble in water, (Fraction 2) copper salts soluble in water but insoluble in methyl alcohol, (Fraction 3) copper salts soluble in both water and methyl alcohol. Proline is isolated from Fraction 3. We have found that large quantities of hydroxyproline are also contained in this fraction.

After removal of the copper, the hydroxyproline is readily separated from proline by its insolubility in absolute alcohol. It can then be isolated as the picrate from the alcohol-insoluble residue. The free hydroxyproline can be obtained by the decomposition of the picrate according to the procedure of Cox and King (2).

Considerable quantities of the pure *l*-hydroxyproline have been prepared in this laboratory by the above procedure.

EXPERIMENTAL

1 kilo of gelatin is hydrolyzed by vigorous boiling for 24 hours under a reflux condenser with 2250 cc. of 33 per cent (by volume) sulfuric acid. After cooling, the hydrolysate is transferred to a 10 gallon stoneware jar and diluted to about 12 liters. 3.9 kilos of solid barium hydroxide are added, and the mixture is stirred until all of the alkali is in solution. The barium sulfate is filtered on a 20 cm. stoneware filter and washed on the filter with three 5 liter

portions of water. The combined filtrate and washings are con-
centrated, *in vacuo*, to about 9 liters. The remaining sulfuric acid
is quantitatively removed by the careful addition of a concentrated
solution of barium hydroxide. The solution is then concentrated,
in vacuo, to approximately 3 liters.

The copper salts are prepared, dried, and extracted with dry
methyl alcohol exactly as described in Town's method.

The solvent is removed from the methyl alcohol-soluble fraction
by distillation. The syrupy copper salts are dissolved in water,
and the copper is removed with hydrogen sulfide. The copper
sulfide is filtered, carefully washed, and the combined filtrate and
washings concentrated, *in vacuo*, to a thick syrup.

This syrup is extracted with 1500 cc. of absolute ethyl alcohol
by heating the mixture on a water bath and stirring it thoroughly.
The insoluble material is filtered and washed with 300 cc. of
absolute alcohol. The filtrate and washings are concentrated,
in vacuo, to a syrup and extracted with alcohol as before. The
mixture is cooled to about 10° and maintained at this temperature
for several hours. A small amount of insoluble material usually
separates. This is filtered, and the filtrate reserved for the prep-
aration of proline.

The insoluble material from the two fractions is combined and
dried in air or in a vacuum oven at 60°. The material is then
weighed, dissolved in 4 times its weight of water, and heated. A
quantity of picric acid equal to the weight of the dried material
is added, and the mixture heated to boiling. 5 gm. of norit are
added, and the solution filtered. The hydroxyproline picrate
crystallizes in long needles as the solution cools. The separation
is completed by keeping the solution at a low temperature for
several hours. The crystals are filtered, washed once with 200 cc.
of ether, and recrystallized from a minimum of boiling water.
The pure hydroxyproline picrate is then dried in air.

The picrate is suspended in 600 cc. of water in a 2 liter separa-
tory funnel, and shaken with 400 cc. of aniline until all of the solid
disappears. The aniline layer is drawn off, and the extraction
repeated with 100 cc. of aniline. The combined aniline extracts
are washed with 500 cc. of water, and the washings are added
to the main aqueous solution. The traces of aniline and a part of
the color are removed by extracting this solution with four 200 cc.

portions of ether.   The solution is then boiled with 25 gm. of norit until it is entirely colorless.   After filtration, the solution is concentrated, *in vacuo*, until crystallization begins.

The crude crystals of hydroxyproline are dissolved in a minimum of hot water, boiled with 5 gm. of norit for 1 minute, and filtered. The filtrate is cooled to room temperature, 10 volumes of 95 per cent alcohol are added, and the solution thoroughly mixed.   The hydroxyproline precipitates in glistening platelets.   The solution is cooled to a low temperature to complete the crystallization.   The crystals are filtered, washed with a little alcohol, and recrystallized from a minimum of boiling water by the addition of alcohol as before.   The hydroxyproline is dried in air or in a vacuum oven at 60°.

Yields of 18 to 20 gm. of the pure *l*-hydroxyproline were obtained.   The acid was found to be free from primary amino nitrogen. The nitrogen by the Kjeldahl method was 10.57 per cent (theory 10.69 per cent).   The specific rotation in water at 20° was −80.54°. Kapfhammer and Eck (3) reported $[\alpha]_D^{20} = -80.6°$.

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THE EFFECT OF FLUORINE ON THE CALCIUM METAB-
OLISM OF ALBINO RATS AND THE COMPOSITION
OF THE BONES*

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INTRODUCTION

It has been shown by the experiments of Forbes and co-workers (8) and Bethke, Kick, Edgington, and Wilder (2) with swine, by Tolle and Maynard (32) with rats, Reed and Huffman (22) with dairy cattle, and Buckner, Martin, and Peter (4) with poultry, that phosphorite minerals used as calcium supplements in animal nutrition may exert harmful effects. These effects may be of a definitely toxic type, such as impaired appetite, disturbances of the general health of the organism, and more specifically a faulty bone and tooth development. Reported analyses of commercial grades of rock phosphate show the presence of from 3 to 4 per cent of fluorine (11). In view of the known toxic properties of this element, it is not surprizing that it is generally believed, though not definitely proved, that fluorine is responsible for the unfavorable results obtained with rock phosphate in animal feeding. In at least two instances effects comparable in nature to those observed by feeding raw rock phosphate have been related to fluorine additions to the ration. The work of Bethke and co-workers (2) at the Ohio Experiment Station shows that the fluorine content of the bones may be increased by the feeding of rock phosphate as well as by sodium fluoride. Tolle and Maynard (32) demonstrated that the feeding of rock phosphate produced an

* The material in this paper is taken from a thesis submitted by F. J. McClure to the Graduate School of the University of Illinois in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Animal Husbandry.

effect on the teeth of rats known to be characteristic of fluorine. It was the purpose of this investigation, therefore, to determine the effects of fluorine on animals, particularly on the metabolism of calcium and on the composition of the bones, and thus to throw some light upon the effects to be expected from rock phosphate when used as a mineral supplement to live stock rations, by reason of its fluorine content.

Effects of Fluorine Reported in the Literature

Numerous cases are on record in which fluorine in one form or another, taken *per os* or by injection in comparatively small amounts, has proved to be extremely toxic and very often fatal. Baldwin (1) and McNally (19) have listed several cases of fluorine poisoning in human beings, Tappeiner (33), Schulz (24), Pitotti (21), Wieland and Kurtzahn (35), Brandl and Tappeiner (3), Schwyzer (26, 27), and others have shown that small dosages of fluorine in various forms and amounts may retard the growth and otherwise impair the normal development and functioning of the organism.

Sollmann, Schettler, and Wetzel (28) have studied chronic intoxications in albino rats, including in their studies sodium fluoride and phosphate rock, being primarily concerned with the probable toxicity of fluorine. Their method consisted in adding a pure form of sodium fluoride to the ration and observing its effect on growth and food consumption. Generally their results show a parallelism between the food intake and the growth of the rats. In three instances, however, the food consumption was normal or above, whereas the rate of growth was below normal. The amount of fluorine in the rations in these cases varied from 0.018 to 0.0452 per cent. These results may be regarded as indicating an effect of fluorine at these levels of intake on the rate of growth of rats. An amount of fluorine equivalent to 0.103 per cent of the ration proved fatal to seven rats within 11 weeks. Six rats received a ration containing 0.045 per cent of fluorine along with a normal ration, both fed *ad libitum*. Three of these rats died by the end of the 5th week. The results show that all the fatalities occurred on the higher level of fluorine feeding, so that it may be concluded that fluorine was the causal factor.

Schulz and Lamb (25) added sodium fluoride to a basal ration which had proved adequate for normal growth and reproduction of rats, in amounts representing 0.0226, 0.0452, 0.0678, and 0.1130 per cent fluorine. The rats receiving 0.0226 per cent fluorine remained normal, those receiving 0.0452 per cent "did not grow as well or as rapidly as the first group." Out of six litters of young in this lot, four were reared, but these grew "at a very slow rate." Rats receiving 0.0678 per cent fluorine grew at about the same rate as the second group. Out of four litters produced, two litters lived. Of seven rats fed 0.1130 per cent fluorine in the ration, all died within 8 to 14 weeks and none of the lot reached 100 gm. in weight. It is evident that the higher amount of fluorine is definitely toxic, but it is difficult to interpret

the effect of the lower amounts in the absence of data concerning the food intake and the rate of growth of the individual rats. Regarding the effect of fluorine on reproduction the authors state that "It appears that an unfavorable effect on reproduction begins at a level of about 0.0250 per cent. sodium fluoride [0.0102 per cent fluorine] in the ration."

In an attempt to duplicate the toxic effects obtained by feeding raw rock phosphate to dairy cattle, Taylor (31) at the Michigan Agricultural Experiment Station has fed several dairy cows varying amounts of fluorine in the form of calcium fluosilicate. The effects noted in this case were a loss of appetite, and a rapid decline in weight. That the teeth of the animals were being affected was indicated by the tendency to lap water. The teeth at this time were observed to be rather narrow and the animal was slightly shear-mouthed. The grinding surfaces of the molars were eventually worn down and the nerves exposed.

The peculiar effect of fluorine on the quality of the teeth of rats was first observed by McCollum, Simmonds, Becker, and Bunting (17). Sodium fluoride to the extent of 0.05 per cent (0.0226 per cent fluorine) was included in an otherwise normal diet for rats. The abnormal growth of the incisor teeth observed appears to be due to changes in structure and hardness of the teeth, which results in a lack of the natural wear which the incisors of the rat exert on each other in occlusion under normal conditions.

The ability of ingested fluorine to modify the structure of the teeth of the rat and dairy cow may be related to its deposition in the teeth to abnormally high levels. That fluorine is a normal constituent of body tissues has been shown by Gautier (9), Zdarek (37), Wrampelmeyer (36), Harms (10), Jodlbauer (12), Sonntag (29), and others. The fluorine content of teeth, judged by the reported analyses, amounts to about 0.2 to 0.3 per cent and is the highest of any body tissue, the average for normal bone would equal about 0.03 to 0.10 per cent. Sonntag (29) found that whereas normal teeth and bones of dogs contained not above 0.3 per cent of fluorine, the bones of dogs fed sodium fluoride contain 1.73 per cent on the moisture- and fat-free basis and the teeth 1.29 per cent on the dry basis. Trebitsch (34) as well as Brandl and Tappeiner (3), have reported the deposition of abnormal amounts of fluorine in various body tissues by continuous small additions of fluorine to the ration. As previously mentioned Bethke and coworkers (2) have furnished evidence of the ability of fluorine, either as sodium fluoride or in the form in which it occurs in rock phosphate, to increase uniformly the amount of fluorine contained in the bones. Pigs fed limestone and steamed bone meal only, had 0.0409 per cent and 0.0231 per cent fluorine, respectively, in the dry fat-free bone. The lots of pigs receiving 30, 60, and 100 gm. of sodium fluoride and 2 pounds of limestone per 100 pounds of basal ration had respectively 0.528, 0.793, and 1.108 per cent fluorine in the dry fat-free bone.

Attempts to demonstrate an effect of fluorine on the composition of various tissues other than an increase in the fluorine content have been generally unsuccessful. Bethke and associates (2) analyzed the bones of

pigs fed sodium fluoride, as well as of those fed rock phosphate, for ash and for the calcium, magnesium, and phosphorus content of the ash, but found no significant differences from the normal bone. Schwyzer (26) observed a slight decrease in the specific gravity of the bones of fluorine-fed rabbits. The reported results of the calcium and chlorine analyses of the bones and the blood of animals fed fluorine by Schwy er (26, 27) show no consistent variations from the normal. However, the blood of rabbits, guinea pigs, dogs, and pigeons fed fluorine according to Schwyzer shows a very high degree of coagulability and an abnormal ratio between the uninuclear and multinuclear leucocytes.

A well demonstrated property of fluorides, is their inhibiting effect on enzyme action. Loevenhart and Peirce (14) studied the effect of sodium fluoride on the action of lipase. Measuring the decrease in the amount of acid produced by the action of the enzyme present in a pancreatic extract on olive oil and ethyl butyrate, these workers found sodium fluoride in dilutions of 1:5000 to have an inhibiting effect of 3 per cent for olive oil and 70 per cent for ethyl butyrate. With clear liver extracts as sources of lipase, dilutions of NaF of 1:5,000,000 inhibited the action of lipase on ethyl acetate 50 per cent. Loevenhart and Peirce tested the effect of a large variety of other salts and found this marked inhibiting effect to be characteristic of fluorides. Lang and Lang (13) have shown that the decomposition of starch by pancreatic juice is checked by the action of sodium fluoride. It appears that fluorine does not affect the action of pancreatic maltase in the hydrolysis of maltose to glucose but does have an inhibitory effect on the conversion of starch to maltose. Clifford (5, 6) has studied extensively the effect of halogen salts in general on enzyme digestion, using as her method of study, in the case of pepsin, the effect of the various salts on the time required for milk to begin to clot. The reaction was inhibited by concentrations of sodium fluoride greater than 0.0144 mol per liter (6).

It is probable that the effect of fluorides on the reactions of muscle and nerve cells may be characteristic of calcium precipitants in general. It is known that muscles taken out of a solution of sodium fluoride, sodium carbonate, sodium acid phosphate, sodium oxalate, citrate, or tartrate and let stand in the air contract powerfully. The nerve placed in the same solutions begins to twitch and goes into tetanus. Robertson and Burnett (23) believe that the above effects are due to a disturbance of the balance of calcium. The effect of the fluorine ion on the chemical changes occurring in the contracting muscle has been investigated by Embden and his school. One rather remarkable effect of fluorine is the preventing of the hydrolysis of the hexosephosphate complex in muscle, a compound formed in the intermediary metabolism of glycogen.

It cannot be said that there is available experimental evidence aside from the work of Bethke and associates (2) at the Ohio Agricultural Experiment Station as to the effect (if any) of fluorine compounds on bone development and calcium metabolism. The most generally known as well as perhaps the most definite effect of the intake of a small amount of fluorine is that produced on the quality of the teeth of rats. Because of similarities in metab-

olism and composition between bone and tooth, an effect of fluorine on bone composition may be at once surmised. The effects of fluorine on enzyme action are of interest, since they might become a factor in the digestion and utilization of rations where fluorine in abnormal amounts is present. How serious the addition of small amounts of fluorine may become as regards the effect on the growth and general health of the organism cannot be definitely stated from the experiments reported in the literature, though the results of practical feeding trials indicate that serious effects may follow, as was the case in the feeding trials with dairy cattle. Small amounts of fluorine have no doubt influenced the food intake and probably the growth of rats fed by Sollmann (28). The results of Schulz and Lamb (25) also indicate that fluorine may retard growth and hinder normal reproduction in rats.

Plan of Experiment

It has not been the purpose of this experiment to obtain an extreme toxic effect of fluorine, but rather to keep the amount of added fluorine at a low concentration, comparable with concentrations that may very well result in the practical feeding of raw rock phosphate as a mineral supplement to live stock, particularly in swine rations. Young growing rats were used as the experimental animals and were fed sodium fluoride in the ration in amounts equivalent to approximately 0.01, 0.03, and 0.06 per cent of fluorine. Two levels of calcium fluoride providing about 0.03 per cent and 0.06 per cent of fluorine were also fed to growing rats. Since the most definitely characterized deleterious effect of the feeding of raw rock phosphate to animals relates to the structure and composition of bone and teeth, the investigation was planned to study primarily the growth, bone and tooth development, and the utilization of calcium of the experimental animals.

Male rats weighing from 60 to 80 gm. were paired so that the pair mates were as nearly the same weight as possible. Each rat was placed in a separate cage with wire mesh bottom and provided with heavy glass cups as containers for water and feed. The paired feeding method was followed throughout the experiment, the purpose being to give to each rat of a pair practically the same amount of feed daily as its pair mate received, the rat voluntarily consuming the least setting the pace for its pair mate. The rats of each pair received the same ration with the exception of fluorine, contained in the ration of one rat of each pair either as sodium fluoride or calcium fluoride. The degree to which the food con-

sumption was kept the same for each pair of rats is indicated in Tables I and II. In six pairs, the total food consumption was the same for each rat of the pair, in nine pairs it differed by 1 to 2 per cent, in one pair it differed by 2.5 per cent, and in one pair (Rats

TABLE I

Effect of Fluorine as Sodium Fluoride and Calcium Fluoride on Growth of White Rats

Rat No.	F present	Ca in ration	Days on experiment	Average daily food intake	Average daily gain in weight	Rat No.	F present	Ca in ration	Days on experiment	Average daily food intake	Average daily gain in weight
	Sodium fluoride fed						Calcium fluoride fed				
	per cent	per cent		gm.	gm.		per cent	per cent		gm.	gm.
1	0.0106	0.154	90	8.22	1.63	19	0.0313	0.315	84	8.54	1.83
2	0.0004	0.148	90	8.13	1.28	20	0.0004	0.294	84	8.62	1.96
3	0.0106	0.154	95	7.98	1.57	21	0.0313	0.315	84	7.49	1.48
4	0.0004	0.148	95	8.17	1.71	22	0.0004	0.294	84	7.49	1.78
5	0.0106	0.154	95	8.16	1.56	23	0.0313	0.315	84	7.97	1.90
6	0.0004	0.148	95	8.14	1.56	24	0.0004	0.294	84	8.05	1.82
7	0.0313	0.341	93	8.88	1.47	25	0.0313	0.315	84	7.78	1.80
8	0.0004	0.341	93	8.81	1.67	26	0.0004	0.294	84	7.83	1.86
9	0.0313	0.341	83	7.78	1.00	27	0.0623	0.615	80	8.45	1.94
10	0.0004	0.341	83	7.69	1.14	28	0.0004	0.569	80	8.48	1.94
11	0.0313	0.341	90	7.61	1.12	29	0.0623	0.615	80	7.34	1.56
12	0.0004	0.341	90	7.55	1.08	30	0.0004	0.569	80	7.29	1.79
13	0.0623	0.596	86	6.65	0.88	31	0.0623	0.615	79	7.55	1.96
14	0.0004	0.634	86	6.64	0.94	32	0.0004	0.569	79	7.52	1.95
15	0.0623	0.596	81	6.29	0.80	33	0.0623	0.615	78	6.76	1.24
16	0.0004	0.634	81	6.36	0.91	34	0.0004	0.569	78	6.68	1.67
17	0.0623	0.596	80	6.10	0.85						
18	0.0004	0.634	80	6.37	0.99						

17 and 18) it differed by over 4 per cent. The rats were weighed every ten days.

The basal ration fed was as follows: sucrose 10, Cellu Flour[1] 4, casein 15, butter fat 10, starch 56, sodium chloride 1, Osborne and Mendel's Ca-free salt mixture (20) 4 per cent. The starch used

[1] A product of the Chicago Dietetic Supply House, Inc. It was found by analysis to contain 37.8 per cent crude fiber.

TABLE II

Effect of Fluorine as Sodium Fluoride on the Calcium Balance of White Rats

The figures are totals for 10 day collection periods.

Period No.	Rat No. and treatment	Ca intake	Ca in urine	Ca in feces	Ca balance	Ca retained
		0.0106 per cent fluorine				
		mg.	*mg.*	*mg.*	*mg.*	*per cent*
1	1. Fluorine	125.6	5.0	37.9	82.7	65.8
	2. Control	117.4	3.7	66.1	47.6	40.5
2	1. Fluorine	130.7	2.5	28.9	99.3	75.9
	2. Control	126.1	2.5	25.9	100.2	79.4
1	3. Fluorine	104.4	5.0	44.0	55.4	53.0
	4. Control	98.2	3.7	51.7	42.8	43.5
2	3. Fluorine	134.5	3.7	20.7	110.1	81.8
	4. Control	132.1	2.5	29.2	100.4	76.0
1	5. Fluorine	115.8	5.0	89.0	21.8	18.8
	6. Control	106.5	5.0	60.1	41.3	38.7
2	5. Fluorine	141.0	3.7	60.2	78.3	55.5
	6. Control	134.5	3.7	48.4	82.4	61.2
		0.0313 per cent fluorine				
1	7. Fluorine	306.1	23.7	82.4	200.0	65.3
	8. Control	314.2	28.7	93.4	192.1	61.1
2	7. Fluorine	323.7	18.7	160.0	145.0	44.7
	8. Control	338.1	15.0	168.1	155.0	45.8
1	9. Fluorine	235.6	16.2	87.7	131.7	58.3
	10. Control	238.3	15.0	73.4	149.9	62.9
2	9. Fluorine	306.7	17.5	161.3	126.9	41.3
	10. Control	295.6	22.5	128.3	138.8	46.9
1	11. Fluorine	247.3	11.2	102.3	133.8	54.1
	12. Control	256.9	8.7	68.9	179.3	69.7
2	11. Fluorine	279.8	11.2	115.0	153.6	54.8
	12. Control	287.4	12.6	152.2	122.7	42.6
		0.0623 per cent fluorine				
1	13. Fluorine	357.5	18.7	180.3	158.5	44.3
	14. Control	402.5	17.5	150.1	234.9	58.3
2	13. Fluorine	405.6	25.2	275.0	105.4	25.9
	14. Control	408.7	21.5	240.2	147.0	35.9
1	15. Fluorine	316.7	18.7	150.3	147.7	46.6
	16. Control	357.4	13.7	143.1	200.6	56.1
2	15. Fluorine	378.6	10.0	294.7	83.9	22.1
	16. Control	388.7	18.7	148.3	221.7	57.0
1	17. Fluorine	326.8	53.5	163.7	109.6	33.5
	18. Control	362.5	12.8	206.3	143.4	39.5
2	17. Fluorine	392.4	25.2	210.2	157.0	40.0
	18. Control	393.1	17.5	289.6	96.0	24.4

was partially dextrinized by pouring boiling hot water over it, with continual stirring. It was then dried rapidly in a hot air bath and ground fine. Merck's casein prepared according to Hammarsten was fed. It contained 0.035 per cent calcium. The rats were fed, in addition to the basal ration, 2 drops of cod liver oil and 0.2 gm. of Northwestern Yeast Company's yeast powder daily.

All the rats were carried through two 10 day balance periods. The first balance periods occurred during the first 6 weeks of the experiment, the second periods during the latter 4 weeks. During these periods the rats were placed in metabolism dishes of the type described by Mitchell (16) for use in determining biological values of proteins. The food intake was controlled and was accurately weighed as in the other feeding periods. Calcium was determined in the rations, feces, and urine by the method of Mc-Crudden (18).

Effect of Sodium Fluoride and Calcium Fluoride on Growth and Food Intake of Rats

From the data in Table I and Chart 1, it is apparent that the three rats receiving 0.0106 per cent fluorine in their ration showed no consistent differences in rate of gain from their pair mates not receiving fluorine. One pair gained at exactly the same average rate, while in another pair the fluorine-fed rat gained more rapidly, and in the third pair the rat not receiving fluorine gained the more. Of the three pairs of rats used in testing the ration containing 0.0313 per cent fluorine, the control rat made the greater gain in two cases, amounting to 0.20 gm. and 0.14 gm. daily. In the other pair the daily rate of gain averaged 0.04 gm. more for the fluorine-fed rat. All of the control rats of the pairs receiving 0.0623 per cent fluorine as sodium fluoride made slightly greater daily gains than did their pair mates. Of the six pairs of rats involved in the two higher levels of fluorine feeding, in five of the pairs the control rat showed a slightly greater average rate of gain than did its pair mate, the fluorine-fed rat. On the other hand, it must be admitted that the greater gain of the control rat in the last pair may have been the result largely of its greater intake of food, which averaged 104.4 per cent of that of its pair mate.

The average daily intake of the rats receiving 0.0623 per cent

fluorine and of their controls was 6.40 gm., while for the pairs in the other two groups, the averages were 8.05 and 8.13 gm., respectively. It seems evident that the highest level of fluorine in
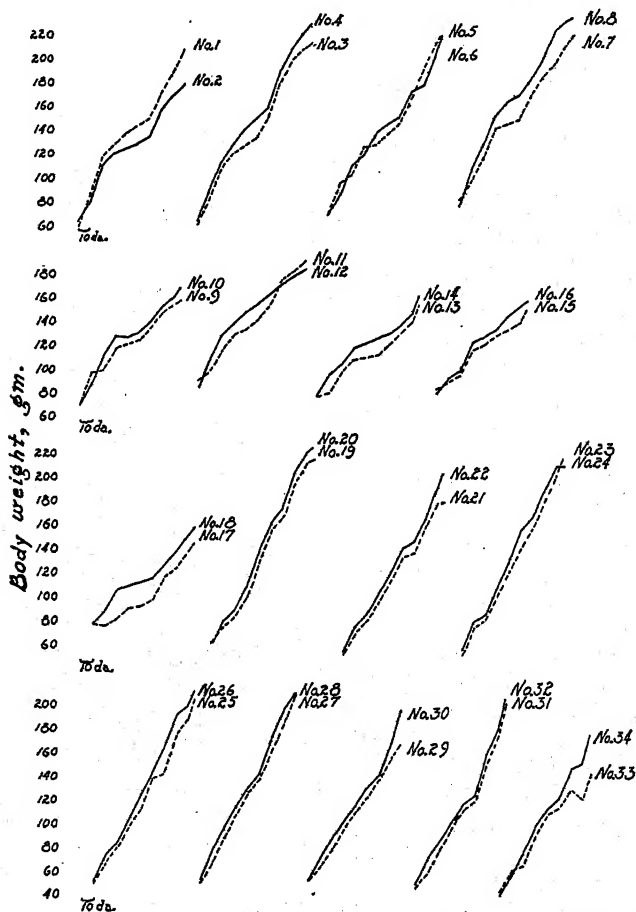

CHART 1. Curves showing rate of growth of pairs of rats. The treatment of each rat of a pair was identical except that fluorine was present in the ration of the odd numbered rat of each pair.

the ration caused a lowering of the amount of food consumed and of the rate of growth induced.

Eight pairs of rats were compared on two levels of fluorine as

calcium fluoride, namely 0.0313 per cent and 0.0623 per cent. The effects on growth and food intake appear in Table I and in Chart 1. In three pairs of rats used in testing rations containing 0.0313 per cent fluorine, the rat not receiving fluorine made a slightly greater average daily gain; i.e., 0.13, 0.30, and 0.06 gm. In the fourth pair the fluorine-fed rat made gains averaging 0.08 gm. greater per day than its pair mate control. However, in the latter pair the control rat exceeded the fluorine-fed rat in body weight throughout the experiment except for the final weight. This relation between control and test rats held throughout the experiment for the other three pairs. The growth curves given in Chart 1, more clearly than the data of Table I, indicate an inhibiting effect of the fluorine ration on growth.

Also at a level of 0.0623 per cent of the daily ration, fluorine as calcium fluoride appears to have retarded growth. In average gain the control rat exceeded the fluorine-fed rat considerably (0.23 and 0.43 gm. daily) in two pairs and made practically the same gain in the other two. But on reference to Chart 1, it is seen that in all pairs at this level of calcium fluoride, except for the final weights on Rats 27 and 28, the control rat maintained throughout the experiment a greater body weight than its pair mate, whether or not it started with such an advantage. Furthermore in the last pair of rats the difference between the control rat and its pair mate widened markedly and progressively in the last half of the feeding period. Thus, with calcium fluoride as with sodium fluoride the indications are definite that the fluorine inhibits growth, entirely aside from its effect on food consumption.

The average daily food intake of Rats 19 to 26 inclusive was 7.97 gm., and that of Rats 27 to 35 inclusive, 7.51 gm. The higher levels of fluorine intake as CaF_2 have thus had little if any effect in lowering the food consumption.

It may be shown by a mathematical analysis of the growth data, more definitely than is possible by mere inspection, that sodium and calcium fluoride inhibited the growth of the rats fed 0.0313 per cent fluorine or more. The average of the fourteen differences in average daily gain between pair mates equals $+0.119$ ±0.025 gm.,[2] the positive sign indicating the greater average gain

[2] The following formula was used to compute the probable error of the mean. $P.E._m = 0.6745 \cdot \sqrt{\dfrac{\Sigma d^2}{N(N-1)}}$. $P.E._m$ = probable error of the mean.

of the control rats.   The standard deviation of this average difference is 0.0365, which will go into the average difference 3.26 times.   From the values of the normal probability integral (7), it may be computed that an average difference of this size or larger would occur by chance only once in 893 trials.   Hence this outcome could hardly have been the result of chance, but must have been a fluorine effect.

In summarizing the effect of fluorides on growth and food intake it may be said with respect to either calcium fluoride or sodium fluoride that at levels of 0.0313 per cent and 0.0623 per cent in the ration, fluorine inhibits growth, entirely aside from any effect on food consumption.   The higher level of sodium fluoride appears to have lowered the food consumption of the rats also; but the corresponding level of calcium fluoride had no apparent influence on food consumption.

Effect of Sodium Fluoride and Calcium Fluoride on Calcium Balance of Rats

A complete record of the calcium intake and output of the rats in the sodium fluoride balance experiment, determined at two different times during the course of the experiment, appears in Table II.   A similar record of the calcium fluoride balance experiment will be found in Table III.   In Table IV are recorded the percentages of the calcium intake retained for each balance period, and the differences in this respect between the rats of each pair.

Since the calcium intake of pair mates was not the same, due to the fact that there were slight differences in the percentages of calcium in the rations, or to differences in the total food intake for the balance period, the percentage retention of calcium seems more significant than the absolute retention.   It is evident that there were no significant differences in the absolute retention of calcium, nor in the percentage retention, between the rats receiving 0.0106 per cent and 0.0313 per cent fluorine as sodium fluoride, and their pair mates.   The average difference (control rat minus fluorine-fed rat) is −2.1 per cent for the pairs concerned with the 0.0106

Σd^2 = the sum of the squared individual deviations from the mean. N = total number of trials in the series.

TABLE III

Effect of Fluorine as Calcium Fluoride on the Calcium Balance of White Rats

The figures are totals for 10 day collection periods.

Period No.	Rat No. and treatment	Ca intake	Ca in urine	Ca in feces	Ca balance	Ca retained
		0.0313 per cent fluorine				
		mg.	*mg.*	*mg.*	*mg.*	*per cent*
1	19. Fluorine	282.9	26.4	108.7	147.8	52.2
	20. Control	267.8	22.5	76.8	168.5	62.9
2	19. Fluorine	364.7	47.9	141.6	175.1	48.0
	20. Control	319.3	50.0	146.2	123.2	38.6
1	21. Fluorine	275.1	28.5	56.9	189.7	68.9
	22. Control	260.5	25.8	62.4	172.2	66.1
2	21. Fluorine	333.3	55.4	110.0	167.9	50.4
	22. Control	300.6	52.0	97.9	150.7	50.1
1	23. Fluorine	256.7	19.0	65.6	172.1	67.0
	24. Control	236.8	28.4	55.7	152.7	64.5
2	23. Fluorine	361.1	48.7	124.2	188.1	52.1
	24. Control	324.9	49.9	114.1	160.9	49.5
1	25. Fluorine	251.4	30.0	56.9	164.5	65.4
	26. Control	236.2	28.9	64.0	143.4	60.9
2	25. Fluorine	331.4	43.1	179.0	109.3	33.0
	26. Control	301.2	49.5	142.5	109.2	36.2
		0.0623 per cent fluorine				
1	27. Fluorine	512.3	39.5	315.4	197.0	38.4
	28. Control	491.1	30.0	331.0	130.1	26.5
2	27. Fluorine	690.0	52.5	495.3	142.2	20.6
	28. Control	645.7	50.4	423.0	172.3	26.7
1	29. Fluorine	519.1	34.1	361.5	125.7	24.2
	30. Control	456.8	41.4	261.0	156.7	34.3
2	29. Fluorine	548.5	44.3	402.1	102.2	18.6
	30. Control	515.9	48.8	322.0	145.0	28.1
1	31. Fluorine	457.2	24.1	299.1	134.9	29.5
	32. Control	414.7	23.0	261.0	130.6	32.3
2	31. Fluorine	673.9	64.0	408.6	131.3	19.5
	32. Control	627.0	51.3	329.9	245.8	39.2
1	33. Fluorine	407.6	30.7	283.4	93.5	22.9
	34. Control	359.3	34.2	182.2	142.9	39.8
2	33. Fluorine	463.3	47.4	315.1	100.7	21.7
	34. Control	477.3	41.0	319.4	116.8	24.5

TABLE IV

Effect of Fluorine as Sodium Fluoride and Calcium Fluoride on the Percentage Retention of Calcium

Period No.	Rat Nos.	Per cent Ca retained		Difference	Period No.	Rat Nos.	Per cent Ca retained		Difference
		Control rats	Fluorine-fed rats				Control rats	Fluorine-fed rats	
0.0106 per cent fluorine as sodium fluoride					0.0623 per cent fluorine as sodium fluoride				
1	1, 2	40.5	65.8	−25.3	1	13, 14	58.3	44.3	+14.0
2		79.4	75.9	+3.5	2		35.9	25.9	+10.0
1	3, 4	43.5	53.0	−9.5	1	15, 16	56.1	46.6	+9.5
2		76.0	81.8	−5.8	2		57.0	22.1	+34.9
1	5, 6	38.7	18.8	+18.9	1	17, 18	39.5	33.5	+6.0
2		61.2	55.5	+5.7	2		24.4	40.0	−15.6
Average......		56.5	58.4	−2.1	Average.......		45.2	35.4	+9.8
0.0313 per cent fluorine as sodium fluoride					0.0623 per cent fluorine as calcium fluoride				
1	7, 8	61.1	65.3	−4.1	1	27, 28	26.5	38.4	−11.9
2		45.8	44.7	+1.1	2		26.7	20.6	+6.1
1	9, 10	62.9	58.3	+4.6	1	29, 30	34.3	24.2	+10.1
2		46.9	41.3	+4.6	2		28.1	18.6	+9.5
1	11, 12	69.7	54.1	+15.6	1	31, 32	32.3	29.5	+2.8
2		42.6	54.8	−12.2	2		39.2	19.5	+19.7
Average......		54.8	53.1	+1.6	1	33, 34	39.8	22.9	+16.9
					2		24.5	21.7	+2.8
0.0313 per cent fluorine as calcium fluoride					Average.......		31.4	24.4	+7.0
1	19, 20	62.9	52.2	+10.7	Average for 0.0623 per cent F......		37.3	29.1	+8.2
2		38.6	48.0	−9.4					
1	21, 22	66.1	68.9	−2.8					
2		50.1	50.4	−0.3					
1	23, 24	64.5	67.0	−2.5					
2		49.5	52.1	−2.6					
1	25, 26	60.9	65.4	−4.5					
2		36.2	33.0	+3.2					
Average.......		53.6	54.6	−1.0					
Average for 0.0313 per cent F.....		54.1	54.0	+0.1					

per cent level of fluorine, and +1.6 per cent for pairs concerned with the 0.0313 per cent level of fluorine. The average percentage of the calcium intake retained by the former pairs is slightly greater than that of any of the other groups of rats. The abso-

TABLE V

Effect of Fluorine as Sodium Fluoride on the Composition of the Bones of White Rats

The results are expressed in per cent.

Rat No.	Femur				Humerus			
	Ash	Ca in ash	P in ash	Ca:P	Ash	Ca in ash	P in ash	Ca:P
0.0106 per cent fluorine								
1	61.81	35.98			59.44	34.19		
2	60.17	34.14	17.92	1.90	63.69	34.27	17.96	1.91
3	57.11	35.53	18.67	1.90	56.51	30.01	17.77	1.69
4	60.21	35.40	17.99	1.97	60.11	31.46		
5	63.17	34.05	17.88	1.90	63.27	31.46	18.36	1.71
6	63.27	33.12	18.21	1.82	60.24	30.93	18.66	1.66
0.0313 per cent fluorine								
7	65.29	33.72	18.81	1.79	65.07	31.92	18.31	1.74
8	63.01	35.15	18.22	1.93	63.49	32.14	17.91	1.79
9	65.12	33.43	18.35	1.82	64.25	30.49	18.91	1.61
10	62.07	34.11	18.37	1.86	61.71	33.45	17.99	1.86
11	63.69	33.66	18.53	1.82	63.50	33.03		
12	63.41	34.66	18.54	1.87	63.11	32.30	17.61	1.83
0.0623 per cent fluorine								
13	63.40	34.85	18.62	1.87	65.37	31.56		
14	62.83	34.63	18.63	1.86	63.48	32.90		
15	62.50	33.35	18.71	1.78	62.79	30.72	18.11	1.70
16	61.91	34.39	17.87	1.92	62.34	33.16	18.90	1.75
17	63.95	33.75			63.83	30.94		
18	61.94	34.69	18.07	1.92	63.80	32.48	18.34	1.77

lute amounts of calcium retained by these rats are, however, smaller than those retained by the pairs testing the higher levels of fluorine intake, no doubt due to the smaller amounts of calcium consumed by them. However, as far as the composition of the bones indicates (see Table V), these smaller amounts in the ration were adequate to meet the requirements of the rats.

With the exception of Rats 17 and 18, Period 2, the fluorine-fed rats receiving a ration containing 0.0623 per cent fluorine in the form of NaF showed smaller percentage retentions of calcium than did their pair mates.   The average difference is 9.8 per cent in favor of the rat not receiving fluorine.   In all but one period out of six, a smaller absolute calcium balance was also obtained for the fluorine-fed rat.   The differences in the amount of calcium retained by the fluorine-fed rat below that of its pair mate varies from 33.8 to 137.8 mg.   That this level of fluorine intake has resulted in a depression of the retention of calcium seems apparent.

An amount of fluorine in the form of calcium fluoride equivalent to 0.0313 per cent of the ration had no apparent detrimental effect on the calcium balance, as shown in Tables III and IV.   In this series of experiments, the amount of calcium retained is less for the control rat in all but one instance than it is for the pair mate receiving fluorine.   Owing to a slightly smaller average calcium content of the rations fed the control rats, the calcium intake of these rats was less than that of their pair mates receiving fluorine. Expressed as a percentage of the calcium intake, the differences in calcium retention between the individuals of a pair are small. All but two of the differences favor the fluorine-fed rat, but the average difference is only −1.0 per cent.

In all but one balance period the rats receiving 0.0623 per cent fluorine as calcium fluoride showed a lower percentage retention of calcium than did their pair mates receiving no fluorine.   The average difference is +7.0 per cent in favor of the control rat. The absolute amount of calcium retained daily is less for the fluorine-fed rat in all but two of the balance periods.

The percentage balance data for the rats receiving 0.0623 per cent fluorine as sodium fluoride or calcium fluoride appear worthy of a statistical analysis.   However, the data for the rats receiving lower levels of fluorine either as sodium fluoride or calcium fluoride do not indicate differences of any significance.   Considering the case of the rats fed 0.0623 per cent fluorine, the average difference in percentage retention of calcium for the same series of comparisons is +8.20 (favoring the control rats), with a probable error of 1.66 and a standard deviation of 2.47.   The average difference is 3.32 times its standard deviation, and would occur, by chance only, once in 1111 trials.   It can be said with practical

certainty that the level of 0.0623 per cent fluorine has lowered the percentage retention of calcium of the fluorine-fed rats.

From these analyses of the data it appears (1) that neither a level of 0.0106 per cent fluorine in the ration as sodium fluoride, nor a level of 0.0313 per cent fluorine as either sodium or calcium fluoride has had an effect on the calcium balances of the rats; (2) that, with a probability equal to practical certainty, a level of 0.0623 per cent of fluorine, either in the form of sodium fluoride or calcium fluoride, has lowered the percentage of the calcium intake that otherwise would have been retained by the experimental rats.

Composition of the Humerus and Femur of the Rat As Affected by Ingestion of Sodium Fluoride and Calcium Fluoride

At the end of from 80 to 90 days, the rats were killed and the two humeri and the two femurs were dissected out. The flesh was removed from the bones as completely as possible, and the bones were dried for 2 days at 96°. The bones were extracted in a Soxhlet extraction tube with 95 per cent alcohol for 18 hours and then with anhydrous ether containing 2.0 per cent alcohol for another 18 hours. After the extraction, the bone residues were dried at 96° for 1 hour and weighed. They were ashed in silica crucibles in an electric muffle furnace for 3 hours and the percentage of ash calculated on the dry alcohol-ether extracted basis. Each sample of bone ash was dissolved *in toto* in 1:1 HCl, and transferred to a 250 cc. flask. The solution was made up to volume with distilled water, and aliquots of this solution were used for the determination of calcium and phosphorus. Calcium was determined by McCrudden's procedure for calcium in feces. Phosphorus was determined by the volumetric method (15), standardized sodium hydroxide being used to titrate the suspension of ammonium-phosphomolybdate precipitate.

From a study of Table V in which the results secured with sodium fluoride appear, there seem to be no differences in the composition of the bones of fluorine-fed and control rats at the lowest level of fluorine intake. However, in all the pairs of rats used to test rations containing 0.0313 per cent and 0.0623 per cent of fluorine as sodium fluoride there is a slightly greater percentage of ash present, in both humerus and femur, for the fluorine-fed rat.

The average difference for the femurs is $+1.46$ per cent in favor of the fluorine-fed rat, and for the humeri $+1.14$ per cent.

On considering the effect of calcium fluoride, as shown by the data of Table VI, here again a slightly greater percentage of ash is present in the bones of rats fed fluoride than in those of their controls, in eleven of the sixteen possible comparisons.

TABLE VI

Effect of Fluorine as Calcium Fluoride on the Composition of the Bones of White Rats

The results are expressed in per cent.

Rat No.	Femur				Humerus			
	Ash	Ca in ash	P in ash	Ca:P	Ash	Ca in ash	P in ash	Ca:P
0.0313 per cent fluorine								
19	63.49				64.03	32.50	17.44	1.86
20	63.34	34.54	18.15	1.90	63.02	32.93	17.84	1.84
21	62.26	34.28	18.49	1.85	62.80	31.77	18.17	1.75
22	62.96	36.49	18.29	1.99	62.99	32.39	18.32	1.77
23	63.26	34.67	18.43	1.88	64.11	33.02	18.35	1.80
24	62.42	34.94	18.63	1.87	63.33	34.10	18.33	1.86
25	62.94	33.08	18.66	1.77	62.94	34.29	18.58	1.84
26	62.73	32.90	18.80	1.75	63.01	32.20	18.27	1.76
0.0623 per cent fluorine								
27	63.86	34.43	18.11	1.90	62.56	34.07	18.02	1.89
28	64.10	36.26	18.59	1.95	63.52	33.84	17.92	1.89
29	64.27	35.16	18.35	1.92	62.71	33.78	18.75	1.80
30	63.47	35.56	18.57	1.91	62.54	33.15	18.56	1.79
31	63.88	34.69	18.28	1.90	64.24	32.54	18.25	1.78
32	63.00	35.30	18.49	1.91	63.40	33.36	18.78	1.78
33	62.92	34.65	18.38	1.88	63.07	34.37	18.71	1.84
34	62.21	36.72	18.46	1.99	63.06	33.22	18.72	1.77

Although the results show a consistent effect of sodium fluoride at levels of 0.0313 per cent and 0.0623 per cent fluorine, a more definite significance may be attached to these results, if the data be interpreted statistically, since the number of trials is not large. The average difference between the ash contents of the humeri and femurs of the rats fed 0.0313 per cent and 0.0623 per cent fluorine as sodium fluoride, and those of their controls, is $+1.305$ per

cent, favoring the fluorine-fed rat. The standard deviation of the differences is 0.991 per cent, and that of the average of the twelve differences (equal to $0.991 \div \sqrt{12}$), equals 0.285. The average difference is therefore 4.57 times its standard deviation, and hence with a degree of probability amounting to certainty it was the result of the directly imposed differential factor, namely, fluorine.

The feeding of calcium fluoride did not give as consistent results on the percentage of bone ash as did the feeding of sodium fluoride. It may be shown by statistical analysis that the data on the ash contents of the bones cannot be interpreted positively as indicating an effect of calcium fluoride. The average difference in the percentage of ash in the humerus and femur of the rats receiving calcium fluoride as compared with their controls is only +0.265 per cent favoring the fluorine-fed rat. The standard deviation of this average is 0.150. The average difference is therefore only 1.77 times its standard deviation. The probability of such a difference (or a larger one) occurring by chance alone is one in thirteen trials. In the case of the femurs alone, the comparison of test and control rats can scarcely be regarded as significant, although out of the eight comparisons six show a greater percentage of ash in the case of the fluorine-fed rat. The average difference here is +0.331 per cent. With such a small number of comparisons the use of "Student's" method (30), devised for the analysis of small groups of paired data, is a fairer basis of interpretation than is the ordinary probability method, based upon the normal frequency curve. The probability as determined by "Student's" method (with $s = 0.543, z = 0.61,$ and $n = 8$) is equivalent to odds of only thirteen to one that the difference is a fluorine effect. It cannot be said, therefore, that calcium fluoride has affected the percentage of ash in the bones of the experimental rats. If such an effect is associated with the feeding of CaF_2 at the levels used in these experiments, it is of such a magnitude that its demonstration will require a considerably larger number of animals than were here used.

A study of Table V shows that there is no consistent difference in the calcium content of the bone ash of the paired rats at the lowest levels of fluorine (NaF) intake. However, at the two higher levels there was a slightly smaller amount of calcium in the ash of the sodium fluoride-fed rats, in ten of the twelve comparisons of the humerus and femur.

The average difference for the femurs is 0.81 per cent in favor of the control rat; for the humeri of the same rats the average difference, possessing the same significance, amounts to 1.29 per cent.

Of the twelve comparisons possible, both femurs and humeri being included, the average difference between pair mates in the percentage of calcium in the ash is -1.05, the negative sign indicating a lower calcium content in the case of the fluorine-fed rat. The standard deviation of this average difference is 0.298. The average difference is 3.53 times its standard deviation. An average difference of this size or greater would occur as a result of chance only, once in 2381 trials. Its significance as a fluorine effect can hardly be doubted.

The effect of calcium fluoride is shown by the data in Table VI; there is a smaller percentage of calcium present in the ash of the femurs of the fluorine-fed rat in all but one of the seven possible comparisons between pair mates. The humeri of the same rats, however, show less calcium in the ash for the fluorine-fed rats in four pairs and more calcium in the remaining four pairs. Thus, with reference to the percentage of calcium in the ash of the bones, as well as to the percentage of ash in the bones, it is evident that the feeding of calcium fluoride has given less consistent results than the feeding of sodium fluoride, indicating a less pronounced effect upon bone composition. The data concerning the percentage of calcium in the ash of the femurs may warrant an analysis by statistical methods.

The significance of the differences obtained may be assessed with a greater degree of certainty by "Student's" method, than is possible by inspection only. The average difference between pair mates in calcium content for the seven possible comparisons is -1.030 per cent, the negative sign indicating a smaller average concentration of calcium in the bone ash of the fluorine-fed rats as compared with their pair mates. The standard deviation of the seven differences is 0.905.[3] The average difference divided by the standard deviation equals 1.14 ("Student's" z), a value of z which for $n = 7$ indicates that an average difference of 1.03 per cent or larger would occur but once in 63 trials, if uncontrolled factors

[3] In "Student's" method, the standard deviation of the sample, s, is computed by the formula $s = \sqrt{\dfrac{\bar{d}^2}{n}}$.

alone determined the results of this experiment. This statistical result represents a fairly high degree of certainty that other than chance factors were operating. Odds of 1 in 30 or 1 in 50 are generally regarded as practically conclusive in biological experiments. It may be concluded, therefore, that the feeding of calcium fluoride at the levels used in these experiments (equivalent to 0.0313 and 0.0623 per cent of fluorine) has lowered the normal content of calcium in the femurs of the experimental rats, although no such effect is evident on the composition of the humeri. Apparently the humerus is less susceptible to dietary fluorine than the femur.

There is no indication that fluorine, either as sodium fluoride or calcium fluoride, had any effect on the phosphorus content of the bone ash. Of eight possible comparisons between pair mates of the results of feeding the two higher levels of sodium fluoride (see Table V), four show a greater amount of phosphorus in the ash of the fluorine-fed rat and four a lesser amount.

The same even distribution of differences in the phosphorus content of the ash occurs for the rats fed calcium fluoride (see Table VI), the humeri only being considered. In the case of the femurs, however, in six of the seven possible comparisons between pair mates, the fluorine-fed rat shows the smaller percentage of phosphorus. The average difference for these seven comparisons is −0.161 per cent and their standard deviation is 0.188 per cent. The average difference is only 0.86 times the standard deviation, which, by "Student's" method of interpretation, indicates a chance occurrence of once in 25 trials. It cannot be said with certainty that the amount of phosphorus in the bone ash has been modified by the presence of either calcium fluoride or sodium fluoride in the ration.

Differences in the calcium to phosphorus ratios in the bone ash for the rats fed sodium fluoride as compared with the same ratios for their pair mates show, in all but one of the eight possible comparisons, a lower value for the fluorine-fed rat, indicating a smaller amount of calcium in proportion to phosphorus in the bone. The average difference for the eight comparisons is 0.0887 with a standard deviation of 0.0774. By "Student's" method of analysis this average difference would be expected to occur by chance but once in 102 trials. The results may be regarded as indicating, there-

fore, a definitely smaller concentration of calcium as compared with phosphorus in the bones of the fluorine-fed rats.

It cannot be said that calcium fluoride has had any influence on the calcium to phosphorus ratio. Of the seven comparisons of the femurs there are three positive differences and four negative differences. Of eight such comparisons based on the humeri, in two cases no difference exists, in four cases the difference is positive, in two negative.

Effect of Sodium Fluoride and Calcium Fluoride on Development of the Teeth of Rats

McCollum, Simmonds, Becker, and Bunting (17) produced marked changes in the development of the teeth of rats by feeding a ration containing 0.0226 per cent fluorine as sodium fluoride. In two rats the effect was apparent within 77 and 97 days respectively. The other rats were on experiment from 201 to 338 days. Second generation rats were fed from 161 to 179 days and showed overgrown incisors. Schulz and Lamb (25) fed the same level of sodium fluoride (0.0226 per cent fluorine) without effect (for how long is not stated). At a level of 0.0452 per cent fluorine as sodium fluoride the teeth of the rats fed by Schulz and Lamb became abnormal.

The rats in the present experiment were fed from 80 to 90 days. No visible effects were noted in the case of those rats receiving 0.0106 per cent fluorine as sodium fluoride. Two rats out of three fed 0.0313 per cent fluorine as sodium fluoride showed the usual abnormal overgrowth of the upper incisors, while the lower incisors are worn down excessively. The teeth of all the rats receiving 0.0313 and 0.0623 per cent of fluorine as calcium fluoride showed the characteristic abnormal development.

It was noted that the teeth of the fluorine-fed rats, besides showing abnormal growth, did not have the peculiar orange tint on the anterior surface characteristic of normal teeth, but were generally a dull white in color.

SUMMARY

The deleterious effects noted in the literature as a result of the use of raw rock phosphate as a mineral supplement have been attributed by investigators generally to its fluorine content. An

attempt has been made, therefore, to study the effect of fluorine on the rate of gain, the calcium balance, and the bone and tooth development of the growing rat.

The results of feeding fluorine to rats indicate that either calcium fluoride or sodium fluoride in the ration at levels of 0.0313 per cent and 0.0623 per cent fluorine inhibit growth, entirely aside from any effect on food consumption. The higher level of sodium fluoride appears to have lowered the food consumption of the rats also, but the corresponding level of calcium fluoride apparently had no such influence.

The calcium balance of the rats was not affected by either sodium fluoride or calcium fluoride at levels of 0.0106 per cent and 0.0313 per cent fluorine in the ration. The results indicate with a probability equal to practical certainty, that a level of 0.0623 per cent of fluorine in the form of either calcium fluoride or sodium fluoride, lowered the percentage of calcium that otherwise would have been retained by the experimental rats.

The composition of the bones of growing rats was distinctly affected by sodium fluoride at levels of approximately 0.03 per cent and 0.06 per cent fluorine. A consistent increase, averaging 1.30 per cent, was evident in the ash content of the bones, and a statistically significant, though not as consistent, a decrease in the calcium content of the ash, averaging 1.05 per cent. The phosphorus content of the ash was not significantly affected, but the ratio of calcium to phosphorus in the ash was depressed.

The results indicate at least a tendency for the same levels of fluorine as calcium fluoride to increase the percentage of ash in the bones. A significant depression of calcium in the ash of the femur, but not of the humerus resulted from calcium fluoride feeding. No definite decrease in the percentage of phosphorus in the ash of the bones accompanied the decrease in calcium occasioned by the calcium fluoride feeding. The Ca:P ratio was not affected.

It may be concluded, therefore, that fluorine, especially when consumed in the more soluble form of the sodium salt, may cause the deposition of an apparently abnormal constituent in the bones, or an abnormal deposition of a non-calcium constituent, possibly a fluoride of a mineral other than calcium, as evidenced by an increase in the percentage of ash in the bones above the normal.

There is also an interference with the deposition of calcium in the bones, brought about by feeding fluorine, a result in agreement with the calcium balance studies, previously described.

The peculiar effect of fluorine on the development of the teeth of rats was again demonstrated in this experiment. A level of 0.03 per cent and 0.06 per cent fluorine in the form of sodium fluoride or calcium fluoride brought about the characteristic abnormalities. Contrary to the results of bone analysis, the insoluble calcium salt was as effective as the soluble sodium salt, in bringing about a change in tooth structure.

Note—After page proofs of this article had been received it was discovered that a definite statement of the calcium supplements added to the basal ration in this experiment had been omitted through oversight. Synthetic mixtures of tricalcium phosphate and sodium fluoride and tricalcium phosphate and calcium fluoride were prepared to contain approximately 4.0 per cent fluorine, the approximate amount of fluorine which is present in commercial grades of rock phosphate. The synthetic mixtures were added to the basal ration to supply the desired percentages of fluorine. Control rations were prepared by adding tricalcium phosphate to the basal ration in such amounts that the calcium contents of control and test rations were approximately the same. The minerals added to the ration replaced an equal amount of starch. All rations were analyzed for calcium.

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STUDIES IN HYPERTHERMIA

II. THE ACID-BASE EQUILIBRIUM IN HYPERTHERMIA INDUCED BY SHORT RADIO WAVES

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INTRODUCTION

In an earlier paper (1) the effect of a hyperthermia induced in human subjects by the high frequency electric current was studied. The effects of hyperthermia produced by short radio waves are reported in the present paper.

The machine utilizing the high frequency electric current was in principle the same as the ordinary diathermy machine in general use, with the exception that it was designed to give an especially smooth high frequency wave to prevent the subject from suffering any disagreeable sensations due to passage of current. In the radio wave machine, the subject rests between the plates without any part of his body's being in contact with the plates so that the waves oscillate through the body from one side to the other. A detailed description of the machine used in our experiments has been given by Carpenter and Page (2). The present study was made to determine whether there was any difference in effect between the hyperthermia induced by radio waves and that induced by the diathermy principle or by the other well known methods of producing artificial fevers such as hot baths or vaccine injections. Carpenter and Page (2) believe

" . . . that the development of heat [by the short radio waves] is due to the resistance of the body to the conduction of current between the surfaces adjacent to the opposed plates. At each alternation of polarity of the plates the corresponding polarities are induced upon the adjacent boundaries of the interposed body and current is conducted through the material for a brief interval. The heating of solutions similar to the blood serum is dependent directly upon their electrical resistance."

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If this explanation be accepted (we do not feel qualified to pass a critical judgment on the various theories) there would be little reason for supposing that the effect of the high frequency current (diathermy) would be different from the effect of the short radio wave. The explanation of Carpenter and Boak (3), however, led us to believe that there were theoretical grounds for a possible difference in physiological effect. These authors state,

"That such a rise in temperature accompanies the passage of the short radio waves through the organism in such a field may be explained in a number of ways, such as that it is conducting induced alternating currents and the heat developed is proportional to the current squared times the resistance of the body. Hosmer's conception is that the heating is due to the increased rate of vibration of the molecules of the cells produced by their alternate attraction to each of the plates in turn. Others consider it analogous to dielectric hysteresis, i.e., a resistance to the changing of the molecules."

Plan of Experiment

The plan of experimentation was essentially the same as that followed in our studies with the high frequency electric current. In addition, a study of the perspiration was made.

Collection of Perspiration—An attempt was made to collect the constituents of the perspiration quantitatively for the entire heating period. The subject was washed with soap and water prior to heating and rinsed with distilled water. He was then wrapped in a sheet and placed upon a cotton blanket, under which was a rubber sheet. He was covered with a cotton blanket. The blankets, sheet, and rubber had previously been rinsed three times in distilled water. The blankets and sheet were dried in a clothes vacuum centrifuge. After the heating the sheets and blankets were washed four times in distilled water. The subject, as well as his hair, was washed with distilled water, gauze sponges being used. All the washings were combined, 1 cc. of concentrated sulfuric acid was added, and the solution was concentrated below 500 cc. Insoluble material was filtered off and washed with dilute acid. The total concentrate was made to a volume of 500 cc. The washings from the rubber were concentrated separately. The amount of perspiration collected from the rubber was not significant. The concentrate gave no precipitate with tungstic acid. Standard methods of blood analysis were used for the determination of non-

protein, urea, ammonia, and creatinine nitrogen and uric acid. Ammonia was separated with permutit.

Analyses—The analytical methods were the same as described in a former paper. The pH, as previously described, was determined by the quinhydrone electrode upon the separated plasma of blood as drawn. The pH was determined at room temperatures. The pH values are reported corrected to the oral temperature of the patient, with the temperature coefficient 0.010. Laug (4) has recently determined the temperature coefficient by the quinhydrone electrode as 0.013 pH per degree for dog plasma. Our value (5) was obtained for two human plasma samples at 25° and 37°. For rabbit plasma we obtained a coefficient of 0.012. The value obtained depends somewhat upon the method of extrapolation of the drifting potential to zero time. For the present we are using our value of 0.010 until more is known regarding the behavior of the electrode the first 15 seconds of the determination.

<div align="center">EXPERIMENTAL</div>

Subjects—We have not felt justified in raising the body temperature of normal individuals above 39°. In the present series, one normal individual was heated on three occasions. The quantitative collection of perspiration was made for this individual. In addition two paretics were subjected to the hyperthermia. One of these showed an initial high temperature of 37.8°. Her temperature was raised to 39.6°. The changes in oral temperature, pulse rate, and respiration rate are given in the protocols.

Blood Volume Changes—With the increase in the oxygen capacity of the blood as a measure of volume change, an appreciable loss in blood volume during the radio wave hyperthermia is noted. Reductions in blood volume amounting to 3.5, 15.0, 6.5, and 7.0 per cent were noted. In hyperthermia induced by the high frequency electric current, the changes in the oxygen capacity approached the error of the analytical determination, increases of 1 and 2 per cent in blood volume being noted in three cases and decreases in 1 and 4 per cent in two others. The data for total hemoglobin are given in Table I.

Blood pH and Alkali Reserve—There was a decided increase in the plasma pH in three of the experiments during the hyperthermia. Shifts of pH from 7.47 to 7.59, from 7.44 to 7.70, and

from 7.46 to 7.55 were noted.   As the normal range for the plasma pH by the quinhydrone electrode is 7.40 to 7.50, the increases are above the upper limits of normal.   In a fourth experiment, the pH remained within normal limits, shifting from 7.40 to 7.44.

TABLE I

Blood Changes Following Rise in Body Temperature

The values for total Hb, HbO₂, and total CO₂ are measured in volumes per cent; those for urea N, non-protein N, cholesterol, creatinine, uric acid, and amino acid N are measured in mg. per 100 cc.

Subject	Date	Oral temperature	Plasma pH	Whole blood findings								
				Total Hb	HbO₂	Total CO₂	Urea N	Non-protein N	Cholesterol	Creatinine	Uric acid	Amino acid N
	1930	°C.										
A.	Apr. 15	36.8	7.47	24.0	13.7	57.9						
		38.9	7.59	24.9	21.0	46.1						
"	Sept. 23	36.9	7.46	21.0	10.6	56.9			151			7.5
		38.9	7.55	22.6	19.0	50.0			151			7.5
L.	July 12	37.5	7.44	20.0	9.1	54.9		31.2		1.3	3.5	8.0
		39.2	7.70	23.5	23.0	37.5		34.5		1.3	3.2	7.0
D.	June 7	37.8	7.40	18.8	7.7	52.1	23.7			1.2	1.8	
		39.4	7.44	20.1	8.6	55.4	25.0			1.5	2.2	

TABLE II

Changes in Distribution of Base Bound by Buffers of Blood During Heating

Subject	ΔBHCO₃	ΔB(Hb)	ΔBP₃	ΣΔB
	mм per l.	*mм per l.*	*mм per l.*	*mм per l.*
A.	−5.8	+5.4	+0.8	+0.4
L.	−10.0	+9.2	+1.7	+0.9
D.	−0.5	+1.1	+0.3	+0.9
A.	−4.0	+4.3	+0.6	+0.9

A correction was applied for the change in blood volume during heating.

This subject had a fever at the time the experimental hyperthermia was induced.   The significance of her condition will be discussed later.

An appreciable fall in the whole blood total CO₂ was noted in

the three experiments in which the plasma pH was markedly increased. A 3 volume per cent rise was noted for the subject whose plasma pH did not change during the hyperthermia. The hemoglobin became highly oxygenated in the three experiments in which the pH rose, and remained unchanged in the experiment in which the plasma pH was unchanged. In order to determine whether the fall in the total CO_2 could be accounted for by a shift of base to the blood proteins in accordance with the increased pH, the base bound by the blood buffers has been calculated. The results are given in Table II. The method of calculation is given in an earlier paper (1). These calculations can be considered only semiquantitatively. In the present instance, they have been complicated by the changes in blood volume. Since the interest is

TABLE III

Quantitative Estimation of Perspiratory Elimination of Subject A

	2.25 hr. period, 36.9–37.6°	2.5 hr. period, 36.9–38.9°
	gm.	*gm.*
Nitrogen as non-protein N....	0.315	0.535
Urea, NH₃-N..................	0.190	0.400
" N......................	0.115	0.160
NH₃-N........................	0.065	0.260
Creatinine N.................	0.010	0.003
Amino acid N.................	0.012	
P............................	Less than 0.5 mg.	Less than 0.5 mg.

primarily whether or not the alkali reserve as a whole is increased, the data obtained during the heating period have been corrected for changes in blood volume. The results indicate a slight increase in the alkali reserve. These results are in harmony with data obtained by us in hyperthermia induced by the high frequency electric current and by Cajori *et al.* (6), who used the electric "bake" and determined the change in alkali reserve by absorption curves. Cajori obtained increases ranging from 0 to 6.0 millimols per liter.

Nitrogenous Blood Constituents—A slight increase in the blood non-protein nitrogen and urea nitrogen during hyperthermia was noted. The increases were comparable to the change in the oxygen capacity. Increases of 1.8 and 1.3 mg. per 100 cc. for urea nitrogen

and 3.3 mg. per 100 cc. for non-protein nitrogen were observed. There was no change and a slight decrease in the amino acid nitrogen for two cases studied. Changes noted in blood uric acid and creatinine were within the error of the analytical determination. There was likewise no change in the whole blood cholesterol. The data are given in Table I.

Nitrogenous Constituents of Perspiration—The high percentage of NH_3 nitrogen as compared with the total nitrogen is perhaps the most interesting observation of the perspiration analyses. The sum of the urea, ammonia, creatinine, and amino acid nitrogen values does not account for the non-protein nitrogen. The data are given in Table III.

Urine pH—In the present study the urine pH was followed at intervals for the preheating, heating, cooling, and recovery periods for three individuals. There was no indication of the urine's becoming alkaline during the period of increased alkalinity of the blood. There was a tendency of the pH to become more acid during the recovery period. The results are given in Table II of the companion paper (7).

<div style="text-align:center">DISCUSSION</div>

Concerning the effect upon the acid-base equilibrium, there is apparently no difference whether the rise in temperature is induced by the external application of heat (hot water or air baths) or by the generation of heat within the body (high frequency current or radio wave). Of fundamental importance is the loss of CO_2 with rise in the blood pH. The fall in the CO_2 content of the blood is readily accounted for by a shift of base to the blood proteins due to the increase in pH.

Cajori *et al.* (6) found no significant change in the blood volume as measured by the oxygen capacity in their studies with the electric bake. We found none in our studies with the high frequency electric current, though the concentration in blood volume following the radio wave hyperthermia was significant. When the high frequency current was used it was necessary to insulate the subject from loss of heat by wrapping him in blankets. In the radio wave series, the effect of the machine was so powerful that the subject was covered with only a thin cotton blanket. It is probable that in the radio wave experiments there was a greater evaporation of

perspiration, while with the high frequency current the subject became bathed in his own perspiration. Since the radio wave hyperthermia is now under investigation for possible clinical use, a warning is sounded as to the change in blood volume it may bring about. In our experiments the subjects drank over a liter of water during the heating and still showed a fall in blood volume.

Donath and Heilig (8) have divided hyperthermias into two classes: those which bring about an increase in the blood amino acid nitrogen with increased nitrogen excretion in the urine and those which do not affect these constituents. According to this classification the radio wave hyperthermia is analogous to the manipulation of the heat centers, which brings about no changes, in contrast to the injection of nucleic acids or vaccines.

The present series of experiments have confirmed our impression that there is little reason for believing that the body is attempting to compensate for the lowered CO_2 tension by lowering the alkali reserve. In experiments with forced breathing and those in which the body temperature is raised very rapidly by immersion in hot water, very alkaline urines and falls in urine ammonia are observed. In our series with the high frequency electric current, some alkaline urines were observed, but they were in no case as alkaline as the blood. In the present series the urine pH at the peak of the heating was 5.0, 6.2, and 5.8. Moreover, the ratio of ammonia to urea in the perspiration increased with rise of temperature and the urine pH became more acid during the cooling period. The latter observation overrules the possibility that the kidneys may have been unable to secrete alkali during the hyperthermia because of the decrease in urine volume.

The findings of the experiment in which the subject had a fever (37.8°) at the time the artificial hyperthermia was induced were unexpected. Her initial plasma pH was within the lower limits of normal. The induced 2° rise in temperature brought about no significant blood changes with the exception of reduction of blood volume. This subject was given a small dose of morphine before the experiment. It is possible that either the morphine or the paretic condition inhibited the normal response of the heat centers. Since another paretic responded normally the condition is apparently not characteristic of paresis. It is not in the scope of the present study to investigate pathological conditions. The

results suggest that studies of hyperthermia induced in various pathological conditions would help materially in elucidating the mechanism of heat regulation.

A study of the nature reported in this paper is possible only with the cooperation of a large number of people.   We are indebted to Dr. H. J. Ullmann for the clinical supervision of the problem, to Dr. R. F. Atsatt, who was the normal subject studied, to Dr. N. H. Brush for supplying us with suitable human subjects for study, and to Miss Ella M. Ottery for the care of the subjects. Mr. Carl Darnell of the General Electric Company was responsible for the radio wave machine placed at our disposal.

SUMMARY

With the exception of a fall in blood volume, which is probably not a direct effect, no difference in effect was noted between raising the body temperature by placing the subject in the field of condenser plates in circuit with a short wave radio transmitter and in raising the body temperature by means of diathermy, warmed air, or hot water baths. Of fundamental importance was the loss of CO_2.   The pH of the blood became more alkaline, there was a shift of bases to the blood proteins and an increased oxygenation of the hemoglobin of venous blood.   No significant change in the non-protein nitrogen constituents of the blood was noted.

No evidence was obtained that the body was attempting to compensate for the condition of alkalosis through the urinary or perspiratory excretions.

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Protocols

T = oral temperature in °C., P = pulse rate, R = respiration rate.

Time	T	P	R	Time	T	P	R	Time	T	P	R
A., male, Apr. 15, 1930				A., male, Sept. 23, 1930				D., female, June 7, 1930			
p.m.				*p.m.*				*p.m.*			
7.15*	36.8	72	20	7.50*†	36.9	80	20	2.30*†	37.8	104	20
7.30	36.8	80	20	8.30	36.7	99	20	3.15	38.0	124	18
7.45†				9.05	38.5	116	24	3.45	38.1	126	
8.00	36.6	76	18	9.30*†	38.9	116	22	4.20	38.9		
8.30	37.0	80	20	9.55	38.4	90	20	5.03*†	39.6	132	24
9.00	37.4	92	22	10.10	37.2	88	20	5.43	38.6	104	24
9.30	38.0			10.40	37.0			6.30	37.6	100	22
10.05	38.6	120	26					7.00	37.6	100	22
10.35*†	38.9	108	26	L., male, July 12, 1930				8.00	38.1	100	22
11.15	38.0	104	16								
12.10	37.0	96	20	2.30*†	37.5	100	20				
				2.55	37.8	100	20				
A., male, July, 1930				3.30	38.4	108	20				
				3.58	38.6	108	24				
8.00*†	36.9	88	20	4.30	39.1	118	24				
8.35	37.1	100		4.35*†	39.2	118	26				
9.00	37.5	92		5.00	39.0	118	24				
9.20†	37.6			5.30	38.4	90	20				
9.45	37.2	92		6.30	37.2	116	20				
				7.30	37.0						

* Taking of blood.

† Beginning and termination of radio wave heating.

STUDIES IN HYPERTHERMIA

III. THE PHOSPHORUS EQUILIBRIUM

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INTRODUCTION

A fall below normal limits in the whole blood inorganic phosphorus was observed in five out of six individuals during an experimental hyperthemia induced by the high frequency electric current (1). Moreover, a decreased urinary output of inorganic phosphorus was observed during the heating and recovery periods. The greatest decrease in urine phosphorus was observed with the greatest fall in blood inorganic phosphorus. Since the blood pH became distinctly more alkaline during the hyperthermia, it was suggested that the observation was an *in vivo* duplication of the *in vitro* conversion of inorganic to organic phosphorus in blood as first demonstrated by Lawaczek (2). There remained two alternative probable explanations, which were capable of experimental investigation. There might be an excessive loss of phosphorus through the perspiration ·or a decreased liberation of inorganic phosphorus by the muscle with increase in metabolism. When the hyperthermia is induced by radio waves, it is quite possible to determine quantitatively the constituents of the perspiration formed during the heating period, an advantage which was utilized in the present study. In our original studies, neither the acid-soluble nor total phosphorus of the blood was determined; nor was the urinary output of phosphorus determined in increments. The rate of phosphorus excretion at the time of the low blood inorganic phosphorus was not known. In the present study, an attempt was made to follow quantitatively the changes in the blood phosphorus both organic and inorganic and

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the urinary and perspiratory output of phosphorus during the pre-, post-, and hyperthermia periods.

Plan of Experiment

As originally planned, hourly single urine specimens were to be collected before and during the heating period. It was not possible to follow through the plan because kidney secretion apparently ceased at the peak of the hyperthermia. The drinking of a liter of water at this time failed to produce urine. The plan was modified so that two collections of urine were made during the heating period. Inorganic phosphorus and total nitrogen were determined in the urine samples. The collection of perspiration is described in the companion paper (3). All the data discussed in the present paper were collected from the experiments recorded in the companion paper. The determination of the blood, inorganic, acid-soluble, and total phosphorus was made according to standard methods upon the same bloods collected for the determination of the acid-base data.

EXPERIMENTAL

Changes in Blood Phosphorus—Analyses for whole blood inorganic and acid-soluble phosphorus and in some cases for whole blood total phosphorus and plasma inorganic phosphorus were made upon blood collected just before the heating was begun and at the time of the maximum oral temperature. The data are incorporated in Table I. The figures in parentheses are values corrected for the change in blood volume which occurred during the heating, as measured by the oxygen capacity. It is apparent that there was in all the cases studied a fall in the inorganic phosphorus during the heating. The values at the time of the maximum temperature are below the lower limits of normal, even before a correction for the change of blood volume is made. An interpretation of the values for the acid-soluble phosphorus and total phosphorus is more difficult. When no correction is made for the change in blood volume a slight increase during heating is observed for all the values but one acid-soluble value, which showed no change. The accuracy of the analytical method for acid-soluble and total phosphorus is generally conceded to be within 5 per cent. The increases observed as measured by an

experimental error of 5 per cent are not significant for the total phosphorus change, but are from 2 to 6 times this error for three of the acid-soluble phosphorus values. When the correction is applied for change in blood volume an increase in the value exceeding the 5 per cent error is only apparent for two of the acid-soluble values. The evidence may be summed up as indicating no change in the total phosphorus during heating, with slight rise in the acid-soluble phosphorus.

TABLE I

Blood Phosphorus before and at Peak of Hyperthermia

| Subject | Temperature | Whole blood | | | Plasma inorganic P |
		Inorganic P	Acid-soluble P	Total P	
	°C.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
A.	36.8	4.0	26.7		
	38.9	2.5	32.5		
		(2.4)	(31.4)		
"	36.9	3.2	27.5	40.0	
	38.9	2.2	27.6	41.6	
		(2.05)	(25.7)	(38.7)	
L.	37.5	3.3	17.5		4.0
	39.4	2.0	23.0		2.45
		(1.7)	(19.5)		(2.08)
D.	37.8	3.0	23.5	33.0	2.9
	39.6	2.9	26.5	36.0	2.5
		(2.7)	(24.9)	(33.6)	(2.34)

The figures in parentheses are values corrected for the change in blood volume which occurred during the heating, as measured by the oxygen capacity.

Urinary Output of Phosphorus and Nitrogen—The urinary output of phosphorus and nitrogen was followed in five experiments for individuals subjected to a hyperthermia induced by radio waves. In one experiment the oral temperature was only raised from 36.9 to 37.6°. A greater elevation of temperature was prevented by blowing warm air over the subject. In this experiment the rate of nitrogen excretion is unchanged as compared with the rate during the 2 hour preheating period and with the 24 hour period. The rate of phosphorus excretion is somewhat diminished as compared with the preheating period. In great contrast to this

experiment a marked decrease in the rate of phosphorus excretion was observed in the four experiments in which the temperature

TABLE II
Urine Findings in Hyperthermia

Subject	Date and time	Oral temperature	Rate of P excretion	Rate of N excretion	Rate of NaCl excretion	pH of urine	Remarks
		°C.	mg. per hr.	gm. per hr.	gm. per hr.		
A.	Apr. 14, 15, 24 hrs.	Normal	33	0.46	0.49	5.6	
	7.00–7.45 p.m.	36.8	45	0.49	0.042	5.7	
	7.45–9.10 "	36.8–37.7	35	0.40	0.030	6.2	6 hr. fast
	9.10–12.25 "	37.7–38.9	6	0.27	0.017	5.0	
	Apr. 16						
	12.25–7.00 a.m.	38.9–37.0	37	0.39	0.039	5.0	
"	July 19, 20, 24 hrs.	Normal	41	0.45			Not fasted
	6.00–7.50 p.m.	36.9	52	0.50			
	7.50–10.30 "	36.9–37.6	33	0.45			
"	Sept. 22, 23, 24 hrs.	Normal	50	0.65			" "
	7.20–8.45 p.m.	36.9					
	8.45–9.45 "	36.9–38.9	44	0.58			
	9.45–10.35 "	38.9–37.2	11	0.53			
L.	July 11, 12, 24 hrs.	Normal	41	0.38		7.6	
	2.00–6.00 p.m.	37.5–39.4–38.3	6	0.10		6.2	6 hr. fast
	6.00–10.30 "	38.3–37.0	46	0.34		5.8	
	July 13						
	9.00–10.00 a.m.	Normal	10	0.32		6.5	
	10.00–11.00 "	"	22	0.56		7.1	Breakfast
	11.00–12.15 "	"	12	0.26		6.8	
D.	June 7						9.00 a.m.
	9.00–12.00 a.m.	Fever	28	0.41		5.8	fruit juice
	12.00–2.30 p.m.	37.8	17	0.24		5.8	5 hr. fast
	2.30–5.00 "	37.8–39.6	3	0.04		5.8	Fasted
	5.00–7.15 "	39.6–37.6	4	0.07		6.0	"
	7.15–10.15 "	37.6–38.1	13	0.12		5.5	"

rise was 2°. In the case of subject A., the fall in the rate of the phosphorus excretion was from 45 to 6 mg. per hour in one experi-

ment, from 50 to 11 mg. in another. The data are given in Table II. There was a slight fall in the rate of nitrogen excretion in one of the experiments with subject A. but not in the other. In the case of subjects L. and D. both the rates of nitrogen and phosphorus excretion fell appreciably during the heating periods. It should be noted that the rates of excretion return to the pre-heating level when the temperature has returned to normal. For subject L. the rate of phosphorus excretion fell from 41 to 6 and returned to 46 mg. per hour. The rate of nitrogen excretion fell from 0.38 to 0.10, returning to 0.34 gm. per hour. For subject D. the rate of phosphorus excretion fell from 17 to 3 mg. per hour during the heating period. During the 2 hour period immediately following the heating period, at which time the oral temperature was still high, the rate remained at 4 mg. per hour. The following period the rate returned to 17 mg. The rate of nitrogen excretion followed a similar course. It is interesting to note that while the rates of phosphorus and nitrogen excretion return to normal in the recovery period, a low rate of phosphorus, but not of nitrogen excretion was observed for subject L. the day following the heating experiment. The data are given in Table II. It was not possible to obtain urine samples at several of the important intervals, so that some of the data include two periods. In the case of subject A., the cooling period and subsequent period are combined in the first experiment, the preheating and heating period in the third experiment. Data for all the periods are thus obtained.

Phosphorus and Nitrogen in the Perspiration—A quantitative determination of the phosphorus and nitrogen in the perspiration was made for subject A. at two different heating periods. Both experiments covered a 2 hour heating period, the one with a slight rise in oral temperature from 36.9–37.6°, the other with a rise of 36.9–38.9°. Only a trace if any phosphorus was eliminated through the perspiration in either case. The total perspiration washings were concentrated to 500 cc. in both experiments. At this dilution the Briggs inorganic phosphate method produced an opalescent solution without development of color when 80 cc. were made to a final volume of 100 cc. To be certain that the other constituents of the perspiration were not inhibiting the development of the blue color, 25 cc. portions of the perspiration solution were subjected to the wet ashing process, with and without

the addition of a known amount of phosphate. Only a faint trace of blue color developed in the samples to which no phosphate was added. The phosphorus excreted in the perspiration was considerably less than 1.0 mg. for the 2 hour heating period in either case. The rate of nitrogen excretion for the temperature range of 36.9–37.6° was 0.157 gm. per hour. With this same rate for the corresponding period in the experiment in which the temperature was raised to 38.9°, the rate of nitrogen excretion for the temperature range 37.6–38.9° is estimated as 0.334 gm. per hour. The total nitrogen excretion for the 2 hour heating period in this experiment was 0.535 gm.

<div align="center">DISCUSSION</div>

Taylor (4) determined quantitatively the nitrogen and phosphorus elimination in the perspiration for two men under normal activity for 28 and 35 day periods. The skin and underclothes were washed daily, the washings being collected and concentrated in a manner similar to our procedure, with the exception that the hair was not washed and the insoluble material was not filtered from the concentrate. A daily elimination of 190 and 160 mg. of nitrogen and 2 and 3 mg. of phosphorus was observed. Taylor concludes that the phosphorus is derived solely from the nuclei of the desquamated epithelial cells and that there is normally no cutaneous elimination of phosphorus. According to our analyses the nitrogen elimination in the perspiration increases from Taylor's normal figure of 8 mg. per hour to 160 mg. per hour for a 1° rise in temperature, and to 330 mg. per hour for a 2° rise in temperature. The fall in urinary nitrogen output during the heating period observed in some of our experiments is therefore readily accounted for by the increased cutaneous elimination. Falls of 13, 28, and 20 mg. per hour were observed in the rate of urinary output in three of our experiments for the periods of maximum temperature. The total nitrogen elimination (renal and cutaneous) is therefore definitely increased during the hyperthermia, an observation in accordance with the increase in metabolism.

The hyperthermia induced by radio waves was identical with the hyperthermia induced by the high frequency electric current in regard to the marked fall in blood inorganic phosphorus and marked decrease in urinary output of phosphorus at the time of

maximum temperature (39°). In the present studies we have shown that no phosphorus is eliminated in the perspiration, that the total blood phosphorus remains unchanged, and that the blood acid-soluble phosphorus may increase slightly. The fall of 1 to 2 mg. per 100 cc. of the inorganic phosphorus, representing a 33 to 50 per cent decrease, should be accounted for by a corresponding rise in the acid-soluble phosphorus if the Lawaczek reaction takes place during the hyperthermia. Since the acid-soluble phosphorus is 5 to 8 times as great as the inorganic phosphorus, a change of 1 to 2 mg. in the acid-soluble value approaches the experimental error of the analytical procedure. The analytical values obtained by us are therefore in harmony with an explanation based on the Lawaczek reaction: due to the increased alkalinity of the blood during heating, there is a conversion of

TABLE III

Balance Sheet for Inorganic Phosphorus Change during Hyperthermia

	Subject D.	Subject L.	Subject A.
	mg.	*mg.*	*mg.*
Decrease in blood P...............	22	70	106
" " urinary P attributed to increased metabolism.........	20–40	41–82	36–72
Decrease in urinary P observed....	60	140	127

inorganic to acid-soluble phosphorus. The question arises as to whether this reaction is confined to the blood alone or whether it also takes place in the tissues.

Kleitman (5) has shown that there is an increase in urinary phosphorus during sleep and a retention during exercise, a 50 per cent reduction in the rate of phosphorus excretion during exercise as compared with the rate for the rest period having been observed. The retention of phosphorus was associated with an increase in metabolism. A part of the decrease in the rate of phosphorus excretion observed during hyperthermia is undoubtedly due to the increase in metabolism which occurs during hyperthermia. However, since the metabolic rate increases only 13 per cent for each degree of rise in temperature, when the rise is gradual as in our experiments, the effect of increase in metabolism in our experiments could not equal that in Kleitman's series, in which the exercise consisted of brisk walking.

Data are available in our present series for estimating the phosphorus balance. The blood volume is assumed to be $\frac{1}{13.5}$ of the body weight at the preheating period. The liberation of inorganic phosphorus by the muscles during the heating period is estimated on the basis of three-fourths to one-half the rate observed during the preheating period. The figures are given in Table III. The

TABLE IV

Changes in the [PO₄≡] and [HPO₄=] Concentration during Hyperthermia.

Subject	pH⁺	Total [PO₄] $\text{M} \times 10^3$ l. H_2O	[HPO₄=] $\text{M} \times 10^3$ l. H_2O	[PO₄≡] $\text{M} \times 10^8$ l. H_2O
H. J. U.	7.47	1.16	1.0	4.9
	7.70	0.68	0.62	5.1
F. E. B.*	7.46	1.00	0.87	4.2
	7.52	0.68	0.60	3.3
	7.52	0.80	0.71	3.9
E. C. W.*	7.48	1.10	0.98	4.9
	7.68	0.90	0.83	6.6
J. E.*	7.54	1.03	0.91	5.2
	7.64	0.87	0.78	5.6
Z. T.*	7.49	1.07	0.93	4.8
	7.55	0.84	0.74	4.4
A.	7.47	1.30	1.10	5.4
	7.59	0.80	0.71	4.6
"	7.46	1.00	0.89	4.3
	7.55	0.66	0.59	3.5
L.	7.44	1.06	0.93	4.1
	7.70	0.55	0.50	4.2
D.	7.40	0.97	0.79	3.3
	7.44	0.93	0.79	3.6

* Hyperthermia induced by high frequency current.

decrease in the urinary output of phosphorus during heating is not accounted for by the increase in metabolism, even on the high estimate of a 50 per cent increase. A satisfactory balance is struck when the change in blood phosphorus is considered. In the case of subject D., there was only a slight fall in blood phosphorus, with only a slight shift in blood pH, an observation in harmony with the other evidence that the fall in blood phosphorus is due to the Lawaczek reaction.

As a matter of interest the $[PO_4^{\equiv}]$ concentration was calculated for the blood before heating and at the maximum rise in temperature, in order to determine to what extent the fall in inorganic phosphorus compensated for the increase in ionization due to the more alkaline pH (see Table IV). An interesting observation is that the $[PO_4^{\equiv}]$ does not change appreciably. The fall in blood inorganic phosphorus might appear to be a defense mechanism to keep the $[PO_4^{\equiv}]$ concentration of the blood constant. The $[HPO_4^{-}]$ concentration becomes appreciably diminished. In our studies in hyperthermia induced by the high frequency electric current a slight increase in blood calcium was observed with the rise in temperature. In this series the $[PO_4^{\equiv}]$ was slightly greater after the alkaline shift in three experiments. The increase in calcium observed in this series cannot be attributed to a change in the $[PO_4^{\equiv}]$ concentration. Since serum is not saturated with respect to $CaHPO_4$, the decrease in the $[HPO_4^{-}]$ observed in all cases, both in the hyperthermia induced by radio waves and high frequency current, could hardly be responsible for an increase in calcium, on the basis of the solubility product of $CaHPO_4$. Further investigation is necessary to determine the significance of the calcium change.

SUMMARY

1. No phosphorus is eliminated in the perspiration during a hyperthermia induced by radio waves.

2. The decrease in the rate of urinary phosphorus excretion is accounted for by the decrease in phosphorus elimination with increase in metabolism and the conversion of inorganic to organic phosphorus in the blood with an alkaline shift of blood pH.

3. The decreased rate of urinary nitrogen excretion is accounted for by the increased nitrogen elimination through the perspiration.

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THE ALKALINE DECOMPOSITION OF SERINE

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INTRODUCTION

A method for the quantitative estimation of arginine is included by Van Slyke (1911–12) in his procedure for the determination of the nitrogen distribution numbers of proteins. The amino acids present in the protein hydrolysate are divided into two groups by means of phosphotungstic acid. The portion precipitated by this reagent is referred to as the basic or diamino fraction and the portion not so precipitated is called the monoamino fraction. Potassium hydroxide, sufficient in amount to give a concentration of approximately 33 per cent, is added to an aliquot part of a solution of the amino acids of the basic fraction and the mixture is boiled for 6 hours under a reflux condenser. The arginine present is thereby decomposed into ornithine, ammonia, and carbon dioxide, and the cystine present is partially decomposed with the evolution of ammonia. At the end of the allotted time, the apparatus is disconnected, water is added, and the ammonia is removed by distillation. The amount of arginine in the original hydrolysate is calculated from the quantity of ammonia evolved, corrections being made for the solubility of arginine phosphotungstate and for the partial decomposition of the cystine.

Plimmer and Rosedale (1925) suggest that for the determination of arginine the division of the amino acids into two groups be omitted. They report that the monoamino fractions from various proteins evolve considerable quantities of ammonia when boiled with alkali. In explanation of this fact, they present the view that the solubility of arginine phosphotungstate is significantly greater than is allowed for by Van Slyke. They conclude: "The arginine value of a protein is the sum of the figures obtained from the di-amino and mono-amino fractions, or the figure obtained

directly, assuming that no other amino-acid behaving like arginine is present in proteins."

In a study of the nitrogen distribution of sericin, a protein of silk which does not yield cystine upon hydrolysis, we determined "arginine" in both the diamino and monoamino fractions.   On the assumption that arginine was the only source of the ammonia evolved, the results indicated that considerably less than one-half of this amino acid had been precipitated by phosphotungstic acid. Further experiments showed that the monoamino fraction contained almost no non-amino nitrogen.   Since three-fourths of the nitrogen of arginine is in the non-amino form, it was evident that the evolution of ammonia from this fraction could not have been due in its entirety to the decomposition of arginine.

In a search for the compound or compounds which gave rise to the excess ammonia, we turned our attention to serine.   The structure of this hydroxy-amino acid suggested that it might be decomposed by alkali with comparative ease.   Furthermore, it is well known that sericin contains a large amount of serine.   A search of the literature revealed a statement by Baumann (1882) that serine is slowly and incompletely decomposed by boiling its solution with barium hydroxide.

Serine was isolated from sericin and treated as though an arginine determination were being made.   Sufficient ammonia to represent a considerable fraction of the nitrogen of the amino acid was obtained.   Other decomposition products were isolated and identified.

It seemed to us important to determine the extent to which this instability of serine affects the existing methods for the determination of the nitrogen distribution numbers of proteins.   It is clear that an attempt to estimate arginine by the method under discussion will lead to an erroneous result whenever serine is present in the solution to be analyzed.   This condition is found in the monoamino fraction, and in the hydrolysate prior to the division of the amino acids, whenever the protein under examination contains serine.   The presence or absence of this amino acid in the diamino fraction depends on whether or not it is at all precipitated by phosphotungstic acid.   According to our results, it is not so precipitated.   The only other point at which the instability of serine to alkali might lead to error is in the determination of

"amide" nitrogen.   It was found that the presence of serine causes at most a very small error at this point.

Except in the case of the modification of the arginine determination suggested by Plimmer and Rosedale, therefore, the instability of serine to alkali cannot be used as a basis for criticism of the methods of determination of the nitrogen distribution numbers of proteins.

<div align="center">EXPERIMENTAL</div>

Preparation of dl-Serine

In the preparation of serine from sericin, we followed, in its general outline, the procedure described by Cramer (1865).   We found it advisable, however, to make changes in certain details. For this reason, and because the journal in which Cramer published is somewhat difficult of access, the following description of the method, as modified, is given.

The protein was hydrolyzed with 25 per cent sulfuric acid for 24 hours.   Barium hydroxide, sufficient in amount to bring the pH of the hydrolysate to 9, was then added and the mixture was allowed to stand at room temperature for an hour.   The solution was next neutralized with sulfuric acid and filtered, and the filtrate concentrated on a steam bath.   Crystals of tyrosine, alanine, and serine appeared successively in the concentrate and were removed, as nearly separately as possible, by filtration.   The serine was recrystallized by adding to its hot aqueous solution an equal volume of alcohol.

The treatment of the hydrolysate with a base was given in order to racemize the serine.   When it was omitted, we were unable to effect the isolation of this amino acid.   This need not surprize us, in view of the fact that l-serine has a much greater solubility in water than has dl-serine.

Analysis of the serine showed 13.2 per cent of nitrogen instead of the theoretical 13.3 per cent; a solution of the amino acid showed no optical rotation.

Effect of Heating Serine in Alkaline Solution

As previously stated, serine was found to decompose into ammonia and other compounds when its solution was boiled with strong alkali.

In Table I are given the results of some experiments which were undertaken to determine the extent of ammonia formation. After each solution containing potassium hydroxide and serine had been boiled for 6 hours, water was added without disconnecting

TABLE I

Effect of Various Bases on Serine

Base used	Concentration of base	Amount of serine added	Time	N evolved as ammonia	Fraction of total N of serine
		gm.	*hrs.*	*mg.*	*per cent*
Calcium hydroxide	Saturated solution	0.482	0.5	0.0	0.0
Magnesium hydroxide	Saturated solution	0.451	0.5	0.1	0.2
Potassium hydroxide	Approximately 14 per cent*	0.586	6.0	5.2	6.7
" "	" "	0.495	6.0	4.0	6.1
Potassium hydroxide	Approximately 20 per cent†	0.161	6.0	1.9	8.9
Potassium hydroxide	Approximately 33 per cent‡	0.286	6.0	14.1	37.0
" "	" "	0.181	6.0	9.1	37.7
" "	" "	0.230	6.0	12.1	39.5
" "	" "	0.289	6.0	14.1	36.6

* The serine was dissolved in 25 cc. of water. 25 cc. of approximately 28 per cent alkali (40 gm. of potassium hydroxide to 100 cc. of water) were added.

† The serine was dissolved in 60 cc. of water. 15 gm. of potassium hydroxide were added.

‡ The serine was dissolved in 50 cc. of water. 25 gm. of potassium hydroxide were added.

the apparatus, and the ammonia was removed by distillation. A pressure of approximately 40 mm. of mercury was maintained during the course of the experiment with calcium hydroxide.

Fig. 1 shows additional results from similar experiments. The plotted curve is typical of a number of such curves obtained with

varying concentrations of different bases. In this series of experiments, the removal of the ammonia was made continuous by means of the passage of a current of ammonia-free air through the boiling reaction mixture.

The following experiments were carried out with the purpose of identifying additional products of the decomposition of serine in alkaline solution.

60 gm. of crystalline barium hydroxide were dissolved in 40 cc. of hot water and the solution filtered directly into a copper flask.
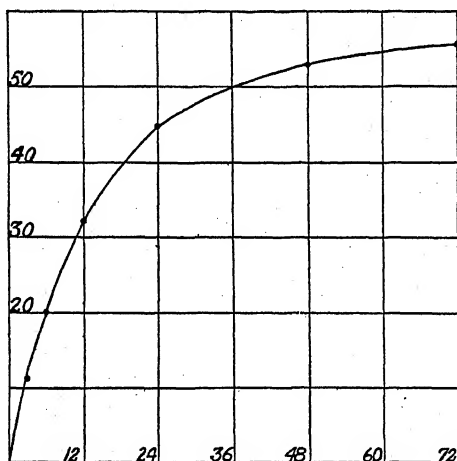
Fig. 1. Decomposition of serine by barium hydroxide. The serine was dissolved in a solution consisting of equal parts of crystalline barium hydroxide and of water. Hours are plotted as abscissæ and the percentages of the total nitrogen evolved as ammonia are plotted as ordinates.

5 gm. of *dl*-serine were then added and the solution boiled under a reflux condenser for 72 hours. In order to prevent the absorption of carbon dioxide from the air the condenser was fitted with a tube containing soda-lime. At the completion of the allotted time, the contents of the flask were quickly filtered on a Buchner funnel and the precipitate washed thoroughly with cold water. This precipitate weighed 1.70 gm. after having been dried at 100°.

Examination of Precipitate—It appeared from microscopical examination that, aside from a few flakes of copper, the precipi-

tate was composed entirely of white crystals which were octahedral in form.   Addition of dilute acetic acid caused only an exceedingly slight effervescence, not more than might be expected from barium carbonate formed during the filtration.   We feel justified, therefore, in stating that no appreciable amount of carbon dioxide was produced from serine during its decomposition.

Dilute hydrochloric acid dissolved the precipitate except for the copper flakes, which were removed by filtration.   The barium was then precipitated by the addition of a very slight excess of dilute sulfuric acid, and the filtrate from the barium sulfate concentrated on a steam bath to a volume of about 3 cc.   Upon cooling this solution in an ice bath, groups of long needles separated which had the appearance of oxalic acid.   They melted at 99°, and when mixed with a known sample of oxalic acid there was no lowering of the melting point.   This identified the original precipitate as barium oxalate.   A mol for mol transformation of serine into oxalic acid being assumed, the barium oxalate recovered represented 16 per cent of the original serine.

Examination of Filtrate—The filtrate from the barium oxalate was chilled and the resulting crop of barium hydroxide crystals was filtered off on a Buchner funnel and washed with ice water. The combined filtrate and washings were then treated with sulfuric acid.   The filtrate from the resulting barium sulfate was concentrated on a steam bath to a small volume.   This concentrate was extracted in a separatory funnel four times with ether.

The combined ether extracts were dried over anhydrous sodium sulfate and the ether removed in a vacuum.   The ether-soluble material thus obtained, when warmed with o-nitrobenzaldehyde and alkali, gave a negative test for pyruvic acid.   The addition of phenylhydrazine produced a beautifully crystalline derivative which was subsequently identified, by means of a mixed melting point with a known sample, as phenylhydrazine lactate, m.p. 103°. While no figures are available, the amount of lactic acid in this fraction must have represented a considerable portion of the original serine.

The solution from which the ether-soluble substances had been removed was evaporated to a small volume on a steam bath and enough absolute alcohol added to make a final concentration of about 70 per cent.   After the solution had stood overnight in the

ice box, there were some needles adhering to the sides of the beaker, and in the bottom a liquid layer which crystallized upon stirring. All of the crystals were filtered off together and washed with 70 per cent alcohol. The filtrate and washings contained 2.6 mg. of nitrogen, about half of which was amino nitrogen. No products could be isolated from this solution, however, and no further hint was obtained as to the nature of its contents.

The crystals, insoluble in 70 per cent alcohol, were found, after thorough drying, to weigh 1.01 gm. Analysis showed the presence of 16.9 per cent of nitrogen in this product, all of which was amino nitrogen. This very high nitrogen content was very strongly indicative of glycine. Accordingly, a portion of the material was dissolved in water and treated with picric acid. After concentration to a small volume and cooling, the solution deposited a crop of yellow crystals. Once recrystallized from water, they melted at 202°, both when alone and when admixed with a known sample of diglycine picrate. This identified one of the decomposition products as glycine.

Sulfuric acid was added to the mother liquors from the diglycine picrate and the picric acid removed by extraction with ether. The resulting solution was then neutralized and treated alternately with sodium hydroxide and α-naphthyl isocyanate. At the expiration of the reaction, the solution was filtered and acidified with hydrochloric acid, whereupon a copious precipitate of a uramino acid was obtained. After many recrystallizations this product melted at 191°, and when mixed with α-naphthyl uramino alanine, m.p. 198°, melted at 195°. Due to the small amount of material available, it could not be purified any further. It seems certain, nevertheless, that alanine also was among the decomposition products.

Pyruvic Acid as an Intermediate Product—Although all tests for pyruvic acid among the end-products of the decomposition were negative, it still seemed probable that it was an intermediate product. This was shown to be the case by the following experiment.[1] 70 cc. of 20 per cent potassium hydroxide solution, containing 1.48 gm. of serine and 2.14 gm. of *p*-hydrazinobenzoic acid, were boiled under a reflux condenser for 25 hours. The solution, after cooling,

[1] We are indebted to Professor H. T. Clarke of the College of Physicians and Surgeons, Columbia University, for suggesting this experiment.

was acidified with dilute hydrochloric acid, care being taken to keep the temperature low. Because of the appearance at this point of a voluminous precipitate of silicic acid the solution was made alkaline with ammonia, and the silicic acid was removed by filtration. Upon reacidification of the filtrate, a crystalline precipitate was obtained which was recrystallized several times. A mixed melting point with a known sample of the p-carboxyphenylhydrazone of pyruvic acid showed the two to be identical. Thus, while pyruvic acid did not appear as an end-product in the alkaline decomposition of serine, it was present as an intermediate product.

Treatment of Serine Solutions with Phosphotungstic Acid

dl-Serine was resolved into its active components by the method of Fischer and Jacobs (1906). The d-serine obtained had a specific rotation of $+6.2°$.

1 gm. of d-serine and 1 gm. of dl-serine were treated separately with phosphotungstic acid under the conditions recommended by Van Slyke (1911–12) for use in the precipitation of the basic amino acids. The mixtures were allowed to stand for 48 hours at 18° and subsequently for several days at approximately 5°. No precipitate formed in either solution.

DISCUSSION

Van Slyke (1911–12) recommends the use of approximately 33 per cent alkali in arginine determinations. To 25 cc. of the solution of amino acids he adds 12.5 gm. of potassium hydroxide. Plimmer (1916) advises the use of a smaller concentration of alkali. He states that in his experiments the concentration of alkali is 20 per cent, but a study of his publications leads us to believe that it is, instead, approximately 14 per cent. As nearly as we can reconstruct his procedure, he dissolves 40 gm. of potassium hydroxide in 100 cc. of water and adds to the solution of amino acids an equal volume of this reagent.

We considered it advisable to obtain data for the production of ammonia from serine under conditions analogous to those employed by Van Slyke and by Plimmer. Accordingly, in the experiments in which potassium hydroxide was used, the concentrations of alkali were approximately 33 per cent, 20 per cent, and 14 per cent, respectively. The amounts of ammonia which were

evolved in these experiments are sufficient to indicate the advisability of separating arginine and serine from one another before the estimation of arginine by the alkaline decomposition method. The percentage error in the determination of arginine in the presence of serine would depend, of course, on the relative amounts of these two amino acids in the solution to be analyzed.

We consider it justifiable to conclude that there is no serine in the basic fractions from protein hydrolysates. Neither *dl*-serine nor *d*-serine gave a precipitate with phosphotungstic acid under the conditions recommended by Van Slyke for the precipitation of the bases, despite the fact that the amount of serine used in each experiment was considerably larger than that which usually arises from the hydrolysis of the sample of protein taken for a determination of nitrogen distribution. There is no reason to believe that *d*-serine and *l*-serine would yield phosphotungstates of unequal solubilities.

Ammonia is usually removed from protein hydrolysates either by saturating with magnesium hydroxide and distilling under atmospheric pressure or by saturating with calcium hydroxide and distilling at a pressure of 30 to 40 mm. of mercury. As shown by the figures in Table I, neither procedure causes a significant decomposition of serine.

That the breakdown of serine under the influence of hot concentrated alkali is a complex process is apparent from a consideration of the nature of the decomposition products. The presence of glycine and alanine among these products indicates that, regardless of the time of reaction, the serine nitrogen would never be completely transformed into ammonia, as these two substances are not appreciably affected under the conditions used (unpublished experiments). This accounts for the shape of the curve in Fig. 1, which indicates that as the time of the reaction was prolonged, the percentage of the total nitrogen evolved as ammonia was approaching a maximum value in the neighborhood of 55 or 60 per cent.

Of the decomposition products identified, it seems that ammonia and pyruvic acid only could possibly be primary ones, and there is no definite evidence that even these two can be regarded as being in that category. The other products are almost certainly the results of secondary reactions, one of which destroys the pyruvic

acid. That the secondary reactions involve oxidation and reduction is attested to by the formation of oxalic acid, which could only be derived from serine by oxidation, and alanine and lactic acid which are reduction products.

It is not unlikely that amino acids other than serine lose ammonia when heated in alkaline solution. Plans have been made to test this assumption and work on hydroxyglutaminic acid is now in progress.

SUMMARY

1. Serine is decomposed when heated in a strongly alkaline solution; among the products of decomposition are ammonia, glycine, alanine, oxalic acid, and lactic acid. Pyruvic acid is an intermediate decomposition product.

2. It is necessary that serine be absent from solutions in which arginine is to be estimated by the method of alkaline decomposition. Our experiments confirm the usual assumption that this absence is secured by the precipitation of arginine with phosphotungstic acid.

3. Serine is not decomposed in significant amount during the removal of ammonia from protein hydrolysates by the methods commonly employed.

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THE PHYSIOLOGICAL EFFECTS OF DIETS RICH
IN EGG WHITE*

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Experiments by Parsons, Smith, Moise, and Mendel (1930), on the physiological effects of various protein-rich diets showed that cooked, dried, commercial egg white is unsatisfactory for reproduction and lactation when fed to adult female rats as either 80 or 20 per cent of a well supplemented diet. Bond (1922) found that reproduction was not optimum with 30 per cent of egg white in the ration, and Mitchell (1925), using egg white to furnish 18 per cent or more of protein in the diet, came to the same conclusion. That the fault, at least on the highest percentages, does not lie in an inadequacy of the proteins of egg white was indicated by the success of Osborne and Mendel (1913, 1913–14), Bateman (1916), Bond (1922), Mitchell (1925), Cowgill (1927), and Hartwell (1928) in inducing satisfactory growth of young rats with the use of egg white, at relatively low levels, as the sole source of protein in the diet.

Chick and Roscoe (1929), in testing the possible value of egg white as the source of protein in the basal diet for the assay of vitamin B (B_1), found, however, that egg white fed as 20 per cent of a relatively purified diet did not maintain the growth of rats to maturity, the growth of some of the rats becoming subnormal even in the first few weeks after weaning. They suggested that some third factor, not identical with the antineuritic nor the antidermatitis factor, present in yeast, is absent from egg white.

Although Boas (1924) concluded that egg white does not contain protein adequate for complete nutrition, she reported in 1927, after further investigation, that egg white, coagulated before drying and properly supplemented with known dietary essentials supports satisfactory growth and nutrition in young rats, but that dried fresh egg white or commercial dried Chinese egg

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white is unsuitable even after cooking for use as the sole protein of the diet of young rats in a similarly constituted ration.   Failure in growth, dermal and nervous symptoms, and death of the rats resulted unless one of certain other food substances was included in the diet with the coagulated egg white which had been first dried before cooking.   Boas (1927) suggested two alternative possibilities: the development of some toxic substance during the drying of the egg white, capable of neutralization by certain protective foods; or the destruction of some hitherto unrecognized dietary essential when drying the egg white before coagulation.   However, neither hypothesis seemed entirely satisfactory.

Norris and Ringrose (1930) have reported a pellagrous-like syndrome in chicks occurring on a diet containing powdered egg albumin.   The manifestations of the disease included sticky eyelids, dermal symptoms, and abnormalities of the mouth, stomach, liver, and kidneys.

Experimental Procedure

Animals—Albino and hooded rats were used as the experimental animals.   The same technique was employed in their care and handling as that described by Parsons, Smith, Moise, and Mendel (1930).

Rations and Their Preparation—Inasmuch as the modifications of Sherman's and of Steenbock's stock rations used previously in experiments on protein-rich diets (Parsons, Smith, Moise, and Mendel, 1930; Parsons, 1930) had proved inadequate for reproduction in successive generations of rats, 5 per cent of butter fat was added to each of these rations, and a further modification of the Steenbock ration devised by House, Nelson, and Haber (1929), in which 2 per cent of yeast and 10 per cent of wheat embryo are included, was used also.   No differences could be detected in the performance of the young rats in respect to these stock rations, when placed on the egg white diets, and hence they are not desig-. nated individually in the results.

Certain of the young rats used in the experiments were produced on the following high protein rations: liver 60 per cent, corn-starch 6 per cent, dried yeast 20 per cent, wheat embryo 10 per cent, salt mixture (Osborne and Mendel, 1919) 4 per cent; casein 64 per cent, corn-starch 2 per cent, dried yeast 20 per cent, wheat embryo 10 per cent, salt mixture 4 per cent; beef powder 66 per cent, dried yeast 20 per cent, wheat embryo 10 per cent, salt mixture 4 per cent.   Liver was also added in varying percentages to the stock rations of certain of the young before weaning.   The egg white rations fed are recorded in Table I.

The egg white employed included both a commercial, dried, Chinese product and egg white from fresh eggs obtained from the University of Wisconsin, Department of Poultry Husbandry. In most of the rations the egg white was fed in a dry powdered form, but for certain of the cooked rations it was not dried.

The commercial dried yeast was assayed for vitamins B and G and found to be potent. The wheat embryo was obtained from a milling company. The liver was prepared by steaming fresh beef liver until it was thoroughly cooked, chopping with a food chopper, drying at room temperature before a fan, and pulverizing.

The egg white was cooked in various ways.

TABLE I

Composition of Rations

Ration	A	B	C	D	E	F	G
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Egg white (dry basis).............	66	66	40	20	66	66	66
Dried yeast......................	20	10	20	20	0	10	15
Wheat embryo....................	10	10	10	10	10	10	10
Sugar...........................	0	10	26	46	0	0	0
Salt mixture*....................	4	4	4	4	4	4	4
Liver (dried beef, cooked)........	0	0	0	0	20	10	5

3 to 5 drops of cod liver oil daily were fed separately.

* Osborne and Mendel (1919).

Boiled Egg White—Dry, commercial, raw egg white was softened in cold water and poured into boiling water to which 3.25 cc. of glacial acetic acid for each 100 gm. of dry egg white were added to bring the solution to pH approximately 4.8, the isoelectric point of egg albumin. Inasmuch as these additions checked the boiling, the mixture was again brought rapidly to the boiling point and boiled for approximately 1 minute.

Cooked for 20 Minutes—Dried egg white was softened in water and was brought to a temperature of 70° in a double boiler without addition of acid, and was then placed in a drying oven for 20 minutes where it reached a temperature of 80°.

Cooked for 3 Hours—Dried egg white was softened in water and was brought to a temperature of 70° in a double boiler without

addition of acid, and was then placed in a drying oven for 3 hours where it maintained a temperature of 80°.

Methods

For water balance studies, the individual cages inclosed in cylinders of sheet tin were set on top of large glass funnels and the urine collected under paraffin oil in large test-tubes. Water and urine were measured at the end of either a 24 or 48 hour period. The intake of water was determined by refilling the drinking bottle to its narrow mouth from a glass cylinder, thus measuring the amount which had been consumed.

The test for occult blood in urine was made by placing some crystals of benzidine hydrochloride with a few drops of urine on a porcelain plate, and adding a small amount of glacial acetic acid and hydrogen peroxide. A strong blue color develops with even traces of blood.

Results

Among the diets rich in protein which have been tested by the author, that high in egg white is the only one exhibiting markedly unfavorably physiological effects on the rat, in striking contrast with the results obtained with casein, dried liver (Parsons, Smith, Moise, and Mendel, 1930), beef powder,[1] and egg yolk.[1] The unfavorable response to egg white was most striking. Healthy young rats from the stock colony rarely survived more than a few days feeding on egg white Ration A. The symptoms noted were loss of weight, lessened activity, an awkward gait in walking, a humped back, meteorism, increased shedding of hair, soiled fur, paws, and tail, closed eyelids, and bloody urine. The rats were often found dead with the jaws firmly closed on the wire mesh of the floor of the cage, or in other attitudes suggesting extreme spasticity. On autopsy the bladder was frequently found to be filled with bloody urine or drops of dried blood were found on the paper under the floor of the cage. The cecum and sometimes the small intestines and stomach were filled with a greenish black, foul smelling mass. If the animal was not autopsied immediately after death, the abdominal organs and peritoneum often showed a

[1] Unpublished data.

distinct discoloration of greenish black. Diarrhea was seldom noticed on any of the egg white rations but it occurred occasionally on the moist rations. Boas (1927), using a lower percentage of egg white, in her extensive experiments on egg white injury, makes no mention of these initial effects during the first weeks on the diet.

TABLE II

*Relative Protection from Egg White Injury during Early Weeks on Ration A (High in Raw Commercial Egg White) Afforded to Rats by Previous Diet**

Group	No. in subgroup		Percentage in subgroup	Distribution of rats in reference to initial weight				
				20-30 gm.	30-40 gm.	40-50 gm.	50-60 gm.	Over 60 gm.
51 rats produced on stock rations	Surviving	5	10	0	0	2	2	1
	Dying	46	90	6	23	8	1	8
73 rats produced on rations containing liver	Surviving	68	93	1	14	16	19	18
	Dying	5	7	1	3	1	0	0
14 rats produced on high beef powder ration	Surviving	6	43	0	2	1	2	1
	Dying	8	57	5	3	0	0	0
24 rats produced on high casein ration	Surviving	8	33	1	2	3	2	0
	Dying	16†	67	8	5	3	0	0

* In some added experiments performed by Fern Stone and Marie Stephens, students in the Department of Home Economics, with slight modifications of Ration A, four rats out of forty-seven, raised on stock rations, survived the initial injury, while forty rats out of forty-seven, produced on high protein rations, survived.

† The ration of nine of these rats contained 56 per cent of egg white and 20 of wheat embryo instead of the 66 and 10 per cent, respectively, in Ration A.

In the course of some studies on various high protein diets during reproduction, a number of young rats were weaned on these rations. Groups of these young placed on egg white Ration A showed a strikingly lower rate of mortality than did those from stock rations. Some of the former rats showed practically none

of the loss of weight and other characteristic signs of the disorder, and other rats recovered quickly. Liver seemed to be the most effective in protection, casein and beef powder less so. The protective capacity of liver was further tested by feeding it to stock mothers and their young from the birth of the young to weaning and for various fractions of this time. The amount of liver varied from 66 per cent to 5 per cent.

In Tables II and III, the influence of these preliminary diets on the performance of young rats when placed on raw egg white Ration A is apparent. Whereas approximately 90 per cent of the rats from stock rations died after a short interval on Ration A, over 90 per cent of the rats survived which had received prelimi-

TABLE III

Extent to Which Decline of Rats Dying on Ration A (High in Raw Commercial Egg White) is Modified by Previous Diet

Rats produced on	No. in group	Age when started, average	Initial weight, average	Maximum weight, average	Minimum weight, average	Days rats lived on egg white, average
		days	gm.	gm.	gm.	
Stock rations..................	46	23	43	42	33	11
Rations containing liver.........	5	19	35	58	30	38
High beef powder rations........	8	21	30	40	27	27
High casein rations.............	16	26	32	42	30	17

nary feedings of liver. In all of the groups, an influence of the body weight of the rat when started on the egg white ration can be seen, the smaller rats tending to die in greater numbers. Among the rats produced on stock rations, however, some individuals died even though the initial body weight was 60 or even 100 gm. This was not true of those produced on high protein diets or fed varying amounts of liver. Some degree of influence of the previous diet is seen even among the groups which died on egg white (see Table III). The forty-six rats from stock rations, which failed to survive, died on an average after 11 days of feeding, with practically no gains in weight; the five from liver rations died after 38 days, with substantial gains in body weight before the final de-

cline.   Their failure to survive seems to have been influenced by the early age at which they were placed on the experimental ration in an attempt to keep the initial weight comparable with other groups of slower growth.   Of three litters fed as little as 5 per cent of dried liver from the 18th to the 21st day all except one rat survived on egg white Ration B, losing weight the 1st day on the ration but fully regaining it on the 2nd.   However, the growth curves of this group showed a temporary plateau after a week or so of good growth, not typical of the curves of the rats which received a more liberal supply of liver before weaning, suggesting that the supply of a protective factor had become exhausted.   The one death in the group occurred at the time of this plateau.

In view of these results it was not surprizing to find that the incorporation of from 5 to 20 per cent of liver in the egg white ration itself, for 1 or 2 weeks at the beginning of the experiment, afforded approximately the same protection from the immediate symptoms of egg white injury as that to be derived from a preliminary period of liver feeding.   1 per cent of liver included in the egg white ration for 1 week appeared to be on the border-line of efficient protection.

The question arose as to whether this initial injury depended upon some quality of raw Chinese egg white not possessed by fresh eggs or by cooked eggs.   Accordingly eggs were procured, the freshness of which was assured, from the Department of Poultry Husbandry, and the dried white from these was fed as Ration A.   Only one rat of a litter of six with body weights of 34 to 36 gm., produced on a stock ration, survived more than 12 days of feeding this fresh dry egg white ration.   Fresh egg white was then cooked in the various ways described under *"Rations,"* dried, incorporated with the other ingredients of Ration A and fed to a group of twelve rats with an average body weight of 34 gm.   All twelve died within a short time.   Chinese egg white acidified and cooked at the boiling point was fed moist, as Ration A, to a group of six rats with average body weights of 80 gm.   Four of the six died in an average of 15 days.   Obviously the injurious effect of egg white is present when either raw or cooked, fresh or commercial egg white is fed in high concentrations.

Cox, Smythe, and Fishback (1929) have pointed out that the injury which Hartwell (1928) reported in young rats fed rations

containing 20 per cent of edestin and low in the vitamin B complex, is strikingly similar to that observed by themselves in rats on cystine-rich diets. The performance of young rats on egg white Ration A in the present experiment is also closely comparable. Both Hartwell and Cox, Smythe, and Fishback found that yeast concentrates were protective against injury. Knott[2] observed that previous liver feeding gave a certain degree of protection to young rats on rations containing 5 and 10 per cent of cystine, but that this protection was less effective than in the case of egg white injury.

The question arose as to whether the injury from egg white might not be due to the high yield of hydrogen sulfide. In Ration A fed to two groups of rats the iron content was varied. In the ration of one group the iron citrate was left out of the salt mixture. In the other, 5 times the concentration of iron citrate in Osborne and Mendel's salt mixture was included, with the idea that this might be effective in reacting with the hydrogen sulfide in the digestive tract. There was no difference in the two groups in regard to the severity of the injury or the occurrence of death.

The previous discussion of survival and protection on egg white has referred only to the initial response of the animals to the ration. The surviving rats which either do not suffer even a temporary decline, or which recover on the egg white diet, usually show, during a later period, striking evidence of nutritional disaster unless complete protection has been secured, as for instance by including 20 per cent of dried liver in the egg white ration (Ration E). The first of these signs of nutritional disorder in the second period is usually a fuzzy, wooly appearance of the coat due to the absence of long hairs. At about the same time, the mouth becomes involved. The first indication of this is either scaly, furrowed red patches at the corners of the mouth, or a bare, sharply demarcated area on the lower lip in the shape of either a sharp wedge or a narrow strip. The whole lower lip becomes bare shortly, the color progressing from a pink to a red and assuming eventually an intense angry hue. The upper lip is slower in showing a change, but the final appearance is the same. Swelling is progres-

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[2] Unpublished data from a thesis presented by Elizabeth Knott in partial fulfilment of the requirements for the degree of Master of Science, University of Wisconsin, 1930.

sive, the lips are distended and have a shiny surface. The bald inflamed areas may spread to the nose, becoming very extensive. No lesions have been observed on the tongue although a cheesy material has been noticed at the base of the lower incisors.

Before the soreness of the lips has progressed far the eyes tend to look watery, and exudate dries on the lids, forming a crust or causing the lids to adhere. When the lids are not closed entirely, the eyeballs appear sunken and glazed, but there is no suppuration as in vitamin A deficiency. There is also almost never an appearance of blood, although the rat scratches and rubs the eyes vigorously enough to produce bald areas around them as in cases of xerophthalmia due to vitamin A deficiency.

While these symptoms are developing, the coat looks more and more unkempt, the hair mats, separates into ribbed bands on the abdomen, and thin or bald areas develop. The period at which baldness occurs, the regions of the body first affected, and the character of the loss of hair vary greatly among individuals, but members of a litter, or litters produced at one time, tend to resemble each other in this respect. At one time, baldness of the back of the head is the most noticeable feature, but at others bareness may appear first most frequently on the thorax, the scrotum, the back, or other regions. The areas are usually symmetrical but may be small and irregularly scattered. The first extensive loss of hair may be at a period of desquamation when large tufts of hair are shed, attached to the dead skin.

Dermatitis sometimes appears early or may be later in developing. The first change noted is in the color of the skin. On parting the hair on the back of a normal young rat, the skin shows a distinct lavender color. In the animals fed egg white, this shifts to a pink or red; or the first alteration noted may be a dirty whitish appearance due to fine scales. Large crusts form on the back, occasionally on the abdomen, thighs, arms, scrotum, or other parts of the body. On healing, these areas become entirely denuded, leaving a delicate new skin, glistening and light pink in color. After a few days a soft fine coating of hair appears on the denuded areas, including the lips, and growth of this proceeds rapidly and the rat soon resumes a normal appearance. The hair on the back, however, although long, remains coarse as the area lacks the fine undergrowth of normal hair.

The ears have never been observed to be affected. The paws and tail however are more or less involved. In some groups of rats, the hair on the backs of the paws, especially the hind paws, becomes matted, and a fine scale appears. In a number of animals the tail becomes scaly also, especially for an inch or 2 inches at the end. Distinct constrictions and ridges may also appear, and the end of the tail may become gangrenous and drop off. These symptoms were often obscured by a coating of the sticky egg white ration on the tail and paws, and so may have occurred in a greater number of instances than was recorded. In most of the severe

FIG. 1. Appearance of rat with pellagra-like symptoms on high egg white Ration A. The eyelids are stuck together. The tail is rough and scaly. The skin, wrinkled and folded, is peeling in large scales. The epidermis of the back is deeply pigmented. The spasticity of the back and legs is shown by the posture. This rat fully recovered when 20 per cent of dried beef liver replaced the dried yeast in Ration A.

cases the wrists become greatly inflamed and somewhat swollen, presumably from the vigorous rubbing of the eyes, mouth, and nose. However, the inflammation usually spreads beyond the surfaces subjected to rubbing, and the entire forearm and paws often become inflamed, swollen, and crusted with scales. As the body becomes emaciated, the skin assumes a characteristic ridged and folded appearance (see Fig. 1).

One striking feature of the dermatitis occurring in almost all of the cases, but which the author has not seen described before for the rat, is a deep pigmentation of the epidermis. Both Sherman

and Sandels (1929) and Goldberger and Lillie (1926) have noted yellow incrustation in pellagrous rats, and Salmon, Hayes, and Guerrant (1928), light brown eschars, but the pigmentation in the present experiment is very dark in shade, tending to a deep brown. It has been noted to occur only on the back, and is bilateral, sharply demarcated, and is usually extensive in area. It is the most persistent symptom at the time of recovery. During the extensive desquamation and formation of new skin of healing it temporarily disappears but soon forms again on the new skin and is only gradually lost, although the rat may appear normal in every other way.

The amount of urine excreted is definitely diagnostic of the advance of the nutritive disorder. With few exceptions, rats severely affected excrete only from 5 to 8 cc. of urine per day, although they may have previously excreted 20 to 30 cc. daily. This is associated with a low water intake and a dehydrated condition of the body, but as the urine excretion is a more constant factor than the water consumed it seems possible that it may be related to the condition of the kidneys. The urine is deep yellow, tan, or green in color, but inasmuch as it is concentrated, it is uncertain whether or not it contains more than a usual amount of pigment. When a cure is instituted an increase in water intake and urine volume is among the first changes noted. Inasmuch as the body weight may increase 7 or more gm. per day at first, a retention of water in the tissues doubtless accounts for this increase to a great extent. With only one exception, obviously bloody urine or occult blood in the urine has not been observed in these rats in advanced stages of the disorder, although as noted before, when the rats die within a short time after being started on the diet, bloody urine is frequently observed.

The rats may continue to grow while a part or all of the symptoms so far described are becoming established, but growth ceases when the rat weighs from 75 to 120 or more gm. and for a period of time the body weight remains practically stationary. During this plateau or when the body weight begins to decline, a very definite nervous disorder usually makes its appearance. The rat is observed to sit in a humped, kangaroo-like position. At first this hump flattens out as the animal walks, but later the hump in the back persists as the rat moves about. Priapism sometimes begins

at an early stage, or may appear later. A progressive spasticity occurs in the legs, particularly the hind legs, first noticeable in the position assumed in climbing the sides of the cage, in which the body is not held close to the cage as in the case of a normal animal, but is held stiffly with the legs extended. The awkwardness in walking then becomes more and more apparent. Finally, sharp jerking motions appear in one or more of the legs as the animal walks. Death is preceded by a rapid loss of weight.

In order to ascertain whether or not the concentration of egg white in the diet bears a relationship to the severity of the nutritional disorder, three litters of six rats each were first protected against initial injury by incorporating 5 per cent of dried beef liver in the stock ration of the mother rats at the birth of the litters, and later when these young reached a body weight of from 40 to 47 gm. they were divided into three groups and fed Rations .A, C, and D containing 66, 40, and 20 per cent of egg white respectively. Inasmuch as Boas (1927) found a variety of food substances, including potato starch, to be protective when incorporated into an egg white ration, starch was avoided in these rations and commercial sucrose was used instead in replacing an equal weight of egg white in the mixtures containing the lower percentages of protein, since this carbohydrate has been used successfully by Burr and Burr (1929) and McAmis, Anderson, and Mendel (1929) in highly purified basal rations. At the end of 24 days on the egg white rations, the growth of the rats was found to be inversely gradated to the percentage of egg white in the rations. For the first 24 days, the rats on Ration A made an average gain of 42 gm.; on Ration C, 77 gm.; on Ration D, 102 gm. Five out of six rats on Ration A showed bareness of the lips, and only one out of six in each of the other groups. The experiment was abandoned at this point because a new lot of dried egg white was proving to have a different effect on all of the rats on experiments, leading to prompt cures in the cases of even those which showed the most pronounced symptoms. A difference in the quality of separate lots of egg white was also the experience of Boas (1927) who attributed the better quality of some samples to partial coagulation during drying at high temperatures. The presence of traces of egg yolk in the dried egg white suggests itself also.

The tail and skin lesions noted by Burr and Burr (1929, 1930) on diets low in certain unsaturated fatty acids seemed so nearly identical with those in the present experiments with egg white (see Fig. 2) that the question arose as to whether the origin of the lesions in the latter case might not be a relative deficiency of those fatty acids, although the daily dose of the 3 to 5 drops of cod liver oil in addition to the 10 per cent of wheat embryo in the ration would presumably furnish the minimum amount necessary. In certain experiments, therefore, lard[3] from oily hogs (Ellis and Isbell, 1926) and linseed oil, both of which substances are rela-
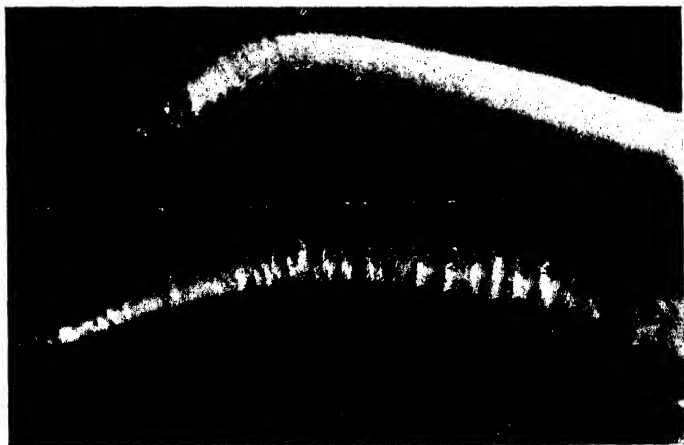
FIG. 2. Appearance of the tails of two rats on high egg white Ration A, showing the typical scaly constructions and ridges and the blackened gangrenous tips which later dropped off.

tively rich in linoleic acid, an essential fatty acid according to Burr and Burr (1930), were fed. Neither raw linseed oil in amounts as high as 30 drops a day nor 10 per cent of lard in the diet delayed the onset of dermal symptoms. 10 drops daily of both linseed oil and commercial lard proved ineffective in cures also.

With both 5 and 10 per cent of liver in the ration (Rations F and G) moderately severe dermal symptoms occurred although they

[3] A small sample of "oily" lard was kindly furnished by W. E. Anderson of the Department of Physiological Chemistry of Yale University.

appeared to be somewhat delayed.   In one case on Ration G even
the nerve symptoms, associated with late stages of the disorder,
were observed.   With 20 per cent of liver (Ration E), on the other
hand, growth was rapid and the skin and hair retained their normal
healthy appearance.   It is evident that the protective level of
liver in rations high in raw commercial egg white lies between
10 and 20 per cent of the ration.   No extent of liver feeding pre-
vious to weaning seemed to insure entire protection from the later
symptoms of injury in spite of the fact that so small an amount
has been shown to protect from the initial injury.

Although by far the greater number of the rats which survived
the first phase of the egg white feeding on Ration A subsequently
showed a very uniform sequence of the symptoms which have
been described above, several exceptional animals from the same
litters as the others appeared to be unaffected by prolonged feeding
of these rations or showed a minor degree of injury.   The reason
for these differences is not fully understood but is thought to be
due to the occurrence of coprophagy in some of the rats.   The
cages which were used throughout have raised wire mesh floors
and no difficulty has ever been experienced with their use in this
laboratory in depleting rats on vitamin B-low rations, death often
occurring in 4 to 5 weeks.   On the egg white rations, however,
the feces although rarely diarrheal, tended to be bulky and sticky
and caught easily on the mesh.   Several of the rats were observed
to eat them.   Sinclair (1930) has reported that rats on "fat-free"
diets show the dermal symptoms described by Burr and Burr
(1929, 1930) only if consumption of feces is prevented.   Because
of the uncertain occurrence of this spontaneous absence of symp-
toms, whatever its explanation, it was found more trustworthy to
produce pronounced symptoms in the rat first, on egg white rations,
and then test the effects of various dietary changes.   In harmony
with the results on prevention with liver in the egg white rations,
it was found that 10 per cent of liver did not effect a cure but, on
the other hand, that practically no stages of dermal or nerve in-
volvement or decline in body weight seemed to be too advanced,
short of actual death, for recovery on 20 per cent of liver.

DISCUSSION

The swiftness of the onset of the first acute symptoms in young
rats weaned from stock diets and fed rations high in egg white

suggests that these symptoms are manifestations of a direct injury from this foodstuff rather than a nutritional deficiency in the usual sense of the term. However, mere digestive disturbances in themselves would seem to be ruled out as an explanation of their origin because of the quantitative nature of the protection afforded by liver even when this is fed previously. These early symptoms strikingly resemble the disorder produced by cystine feeding, and there is the further analogy that cystine injury has been prevented by the use of yeast concentrate in the ration (Cox and Hudson, 1930).

The later dermal and nervous manifestations of more insidious onset, which occur on egg white Ration A after an apparent recovery from the first acute disorder, have no counterpart in any symptoms so far reported from cystine feeding. Injury, however, rather than a simple deficiency is indicated by the fact that the symptoms occur less early and in milder form with 20 or 40 per cent of egg white in the ration than with 66 per cent. The assumption must be made in drawing this conclusion that no protective factor is introduced with the sucrose used in diluting the egg white. On the other hand, the symptoms are as clear cut and uniform as for most of the deficiency diseases and may be prevented or cured by a definite concentration of liver in the ration. The resemblance of these symptoms of slow onset to those of pellagra is most striking and cannot be ignored in spite of the fact that 20 per cent of a dried yeast rich in vitamin G is included in the ration, and that dried egg white itself is known to be potent in the antidermatitis factor (Aykroyd and Roscoe, 1929). Complex dietary relationships have been shown in the past to be responsible for various anomalous results in nutrition experiments. The injurious effects on reproduction of adding ferric chloride (Waddell and Steenbock, 1928) or a high percentage of lard (Mattill, 1927; Evans and Burr, 1927) to otherwise adequate rations have been explained on the basis of a destruction of vitamin E in diets apparently containing an adequate amount of this vitamin. While unquestionable instances of unusual vitamin requirements on a given diet, or of lessened absorption or utilization of a vitamin, cannot so readily be cited, such conditions are not inconceivable. They might perhaps furnish as tenable hypotheses as postulating a new deficiency disease to account for the symptoms which so strikingly resemble pellagra in the present

experiment.   Norris and Ringrose (1930) attribute the pellagrous-like syndrome in chicks on an egg albumin ration to the intense requirement of this species for vitamin G.   The results of Under-hill and Mendel (1925, 1928) on the dietary deficiency canine disease resembling black tongue have been interpreted to indicate that pellagra may originate in more than one way.   Reader (1930) has suggested that pellagra may be due to a combined deficiency of vitamin B_2 and a third as yet unidentified component of the vitamin B complex.

It is of interest to note that work by Sullivan and Dawson (1920–21) suggested that the sulfur metabolism is abnormal in acute cases of pellagra.   They considered that the decrease in the normal sulfocyanate content of the saliva and urine of patients with active pellagra might be interpreted as an indication of the relative failure of detoxification by means of the -SH radical.

The results herein recorded confirm Bateman (1916) in suggest-ing caution in the use of as large quantities of raw egg white in invalid feeding as has been the common practice in many hospitals in the past.   Furthermore the fact that many cases of eczema in children have been observed to be associated with a sensitization to egg white raises the question as to whether the early introduc-tion of egg white into the somewhat restricted dietary of the child may perhaps need to be safeguarded with protective foods in somewhat the same way as its introduction into the diet of the rats in the present experiment.

SUMMARY

A nutritional disorder and death speedily result in the case of the majority of young rats weaned from stock rations when fed diets high in egg white, either raw or cooked, commercial, dried or fresh.   The rats may be protected from these initial symptoms by even as little as 5 per cent of dried liver fed for 3 days before the beginning of the egg white diet. .

On a ration containing 66 per cent of dried egg white, either raw, Chinese, or from raw fresh eggs, some symptoms later de-velop in the rats surviving the first few weeks which strikingly resemble those of pellagra even though the rations contain 20 per cent of potent dried yeast.   These symptoms may be prevented or cured by 20 per cent of dried beef liver but not by 10 per cent.

Lard and raw linseed oil are not 'effective either in preventing or in curing the symptoms.

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STEROL CONTENT AND ANTIRACHITIC ACTIVATIBILITY OF MOLD MYCELIA*

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INTRODUCTION

During the last few years great interest in the study of the sterols
has been manifested. The great stimulus in the field of sterol
research is the result of the discovery that the sterols, as for
example ergosterol, yield vitamin D on irradiation with ultra-violet
light. Because of this relation to vitamin D, many questions have
arisen regarding the distribution, isolation, identification, and
properties of the naturally occurring sterols. Although there are
numerous references in the literature to the occurrence of sterols
in ergot, yeasts, mushrooms, and higher fungi, only a few studies
have been reported which deal with the presence of sterols in
molds.

Gérard (1) isolated a sterol from *Penicillium glaucum*, grown on Raulin's
medium, which melted at 135°, had a specific rotation of −143.3°, and gave
color reactions similar to Tanret's ergosterol. Later (2) he also reported
the presence of a sterol in *Mucor mucedo*. He grew the mold on a synthetic
inorganic medium containing lactose, but was unable to isolate enough of
the sterol to determine its physical constants. Rémond and Lassalle (3)
found that *Penicillium glaucum* grown on bread uses up the carbohydrate
of its medium and transforms it into fatty acids, lipoids, and "cholestérine."
Gayral (4), in his studies on the influence of different radiations on the
nutrition of *Aspergillus niger*, made quantitative determinations of "phyto-
stérine." He found that the dry pads contained 0.5 to 0.6 per cent of this
material. In their paper on yeast ergosterol, Reindel and Walter (5)

briefly mention that they also prepared ergosterol from the fat of a *Mucor sp.* This ergosterol was identical in properties with that obtained from yeast fat. Heiduschka and Lindner (6) made quantitative colorimetric determinations of the ergosterol content of ten fungi, including three molds. They found that the dry matter of *Dematium pullulans, Penicillium glaucum,* and *Aspergillus oryzæ,* Ahlberg, contained respectively 0.30, 0.75, and 0.46 per cent ergosterol. The authors believe that such factors as temperature, period of incubation, and type of nutrient media influence the ergosterol content of the fungi more than the species. Sumi (7) isolated 0.8 gm. of crude ergosterol from 1 kilo of *Aspergillus oryzæ* spores. Takata (8) reports the isolation of 2.8 gm. of sterol from 1 kilo of dried *Aspergillus oryzæ* mycelium. The purified colorless needles had a m.p. of 147–148° and gave the characteristic ergosterol color reactions. Barber (9) mentions the isolation of small quantities of crude sterol from a *Penicillium sp.* grown on Czapek's solution. Bills and his coworkers (10), in their study of the factors determining the ergosterol content of fungi, including eighteen molds, found that a neutral or slightly alkaline medium, an abundant air supply, and certain concentrations and combinations of nutrient salts were conducive to vigorous growth and relatively large ergosterol production.

From this brief review it is evident that our knowledge is very meager in regard to the number and kind of sterols found in molds. The work that has been done on their identification is more or less conflicting. Even less is known regarding the effect of such factors as oxygen supply, temperature, reaction, and nutrients on the production of sterols. Answers to many of these questions will be obtained when detailed information is available regarding the metabolic processes of these molds.

The present paper reports the study of a large number of molds which were tested with respect to (1) their ability to grow on a synthetic culture medium, and (2) their antirachitic activatibility. On the basis of this preliminary survey, eleven molds were chosen for growth in mass cultures and the effect on their sterol content of certain factors were studied.

<div align="center">EXPERIMENTAL</div>

Cultures and Medium—The molds used in this work were either stock cultures that had been carried in the laboratory for a number of years or cultures obtained for this work from investigators in this and in foreign countries. In most cases the genus of the culture was known and in a small number of cases the species was identified. All of the cultures on which sterol determinations were

made were checked as to their purity and identity at the beginning and end of the work.

The synthetic culture medium on which the molds were grown was essentially that used by Peterson, Fred, and Schmidt in their study of the fermentation of pentoses by molds (11). A slight modification was made in that 0.05 gm. of $ZnSO_4 \cdot 7H_2O$ was added. The composition of the medium, designated later as Medium G.S., is as follows:

NH_4NO_3	10.00 gm.
KH_2PO_4	6.80 "
$MgSO_4 \cdot 7H_2O$	5.00 "
$FeCl_3 \cdot 6H_2O$	0.16 "
$ZnSO_4 \cdot 7H_2O$	0.05 "
Glucose	40.00 "
Distilled water	1000.00 cc.

The individual salts were made up in quantities sufficient to cover a series of experiments. Each stock salt solution was kept in a separate flask and combined with the others as needed.

Preliminary Experiments—A preliminary survey was made of the growth of 55 mold cultures on 100 cc. of the glucose-salts medium contained in 500 cc. Erlenmeyer flasks. Four flasks were used for each culture. These cultures were incubated for 10 days at 28°. Of the cultures thus tested twenty-three showed good growth. The results appear in Table I. The yields of pad varied from 0.29 gm. in the case of the very filamentous *Rhizopus nigricans* to 1.40 gm. for the *Hormodendrum sp.*

Biological Test of the Antirachitic Potency of Certain Molds—Since molds are known to contain ergosterol, it is possible to obtain an index of the relative amounts of this sterol that are present by feeding some of the irradiated pad to rachitic rats.

The dried mold pads obtained in the above preliminary experiments were ground to pass a 100-mesh sieve and of this finely ground material 0.5 gm. was spread over the bottom of a pie tin $7\frac{3}{4}$ inches in diameter. The material was irradiated for 15 minutes under an Alpine Sun lamp at a low intensity and at a distance of 18 inches. To insure proper irradiation, the material at the end of this period was redistributed and further irradiated for 15 minutes. Proper quantities of the irradiated pad were thoroughly incorporated with 50 gm. of rickets-producing Ration 2965

TABLE I
Growth and Antirachitic Potency of Certain Fungi

Trial No.	Fungus	Weight of dry pad per 100 cc. medium*	Line test† 50 mg. level	Line test† 10 mg. level
		gm.		
1	*Aspergillus sp.*, Culture 4	1.08		
2	" " " 43	1.06		
3	" *niger*, Culture 1	1.10	+++ +++++	+++
4	" " " 59	0.92		
5	" *oryzæ*, " 1	1.29	+++ +++++	+++
6	" " " 2	1.01	+++++ +++++	+++
7	" " " 192	0.70		
8	" " " 965	0.60		
9	*Clitocybe multiceps*	1.02	++++	+++
10	*Coprinus radians*	0.59		++
11	*Didymopsis sp.*	1.33	+++++ +++	+
12	*Fusarium sp.*, Culture 20	1.23	++++	+++
13	" " 23	0.82		
14	*Hormodendrum sp.*	1.40	+++++ ++++	+
15	*Penicillium sp.*, Culture 9	1.32		
16	" " " 29	0.98		
17	" " " 111B	1.19	+++ ++	−
18	" *expansum*	0.90		
19	" *janthinellum*	0.80		
20	*Rhizopus sp.*, Culture 25a	0.50		
21	" *nigricans*	0.29	+++++ ++++	+++
22	*Trichoderma sp.*, Culture W.	1.02	++ ++	+++
23	" *lignorium*, Culture 1372	0.64	+? +?	−
24	*Boletus clintonianus*		+++++	?
25	*Clitocybe multiceps*	Mushrooms		+?
26	*Marasmius oreades*	gathered	++++	++
27	*Hypholoma incertum*	in the		+++
28	*Secotium acuminatum*	open	+++++	++

* Incubated at 28° for about 10 days. Pads dried at 65° for 1 day.

† Complete healing +++++; almost complete healing ++++; wide line or narrow metaphysis +++; medium line or medium metaphysis ++; narrow line +; specks of calcification or indication of a line ?; wide rachitic metaphysis −.

(Steenbock and Black (12)), and fed to rachitic rats that had been on this ration for 3 to 4 weeks. The irradiated pad was fed at levels of 50 mg. and 10 mg. per rat during the test period of 7 days. At the end of 10 days the rats were killed and the standard line test was made. Also five kinds of mushrooms, gathered in the open, were similarly tested for their antirachitic activatibility. As recorded in Table I, eight of the molds and three of the mushrooms showed distinct healing of rickets even when fed at a level of 10 mg. This shows that these fungi are highly potent materials.

Growth of Molds in Quantity—With these preliminary results for guidance, it was decided to grow certain of the molds in quantity so that sufficient material would be available for sterol determinations. The molds chosen for more extended study represented the principal species which grew well on the synthetic medium and gave good results in the animal experiments. The molds were grown in six $12'' \times 22'' \times 2\frac{1}{2}''$ and five $11'' \times 19'' \times 3''$ tin pans fitted with galvanized iron covers. The latter pans were made in such a manner that the covers rested on shoulders at the ends of the pans, thus keeping them slightly above the sides of the pans and increasing the supply of air. The pans were wrapped in paper to reduce the chances of contamination due to air currents. The eleven pans, each containing 2 liters of medium, were autoclaved at 15 pounds pressure for 20 to 30 minutes. After cooling, they were seeded with a well sporulated inoculum. The latter had been grown in round, 1 liter bottles containing 100 cc. of Medium G.S. Usually the mycelium of one such bottle was sufficient to inoculate three pans. The pans were incubated until the mold pads began to sporulate—about 10 days. After this period of incubation, the pans containing the mold pads were again autoclaved at 15 pounds pressure for 20 minutes. The pads were then removed from the pans, washed, dried at 65°, weighed, ground in a drug mill, and saved for future use.

The weight of dry pad per pan of the eleven molds grown in quantity varied from 7.6 to 77.0 gm. The majority of the pads weighed from 15 to 20 gm. each.

Factors Affecting Weight of Mycelium—With *Aspergillus oryzæ*, Culture 2, such factors as temperature, period of incubation, quantity of medium, wrapping of pans, glucose content of the medium, and supplementary organic material in the basic medium

were studied in a preliminary manner to ascertain the requirements of practical working conditions. Table II shows the in-

TABLE II

Some Factors Affecting the Weight of Mycelium Produced by Aspergillus oryzæ, Culture 2

Factor	Trial No.*	Area of pan†	Glucose content of Medium G.S.	Period of incubation at 28°	Weight of dry pad per pan	
		sq. in.	*per cent*	*days*	*gm.*	*gm. per sq. in.*
Period of incubation	1	209 (2 pans)	4	9	18.95	0.091
	2	209 (5 ")	4	47	5.20	0.025
Concentration of glucose in medium	3	209 (1 pan)	2	10	7.21	0.027
	4	264 (1 ")	2	10	7.00	0.033
	5	209 (1 ")	4	10	8.86	0.042
	6	264 (1 ")	4	10	9.31	0.035
	7	209 (1 ")	6	10	17.81	0.085
	8	264 (1 ")	6	10	16.78	0.064
	9	209 (1 ")	8	10	28.79	0.138
	10	264 (1 ")	8	10	29.97	0.113
	11	209 (1 ")	10	10	28.00	0.134
	12	264 (1 ")	10	10	30.21	0.114
Organically supplemented medium	13	264 (4 pans)	4‡	10	14.5	0.055
	14	264 (4 ")	4§	10	17.5	0.066
	15	264 (4 ")	4‖	10	15.2	0.058

* In Trials 1 and 2 pans were unwrapped; in Trials 3 to 15 pans were wrapped in paper.

† Pans with an area of 209 square inches were constructed with shoulders, thus raising the covers and allowing greater access of air. Those with an area of 264 square inches had no raised covers. Each pan contained 2 liters of medium.

‡ Each 2 liters of Medium G.S. contained 500 cc. of 10 per cent aqueous malt sprouts extract.

§ Each 2 liters of Medium G.S. contained 1000 cc. of 10 per cent aqueous malt sprouts extract.

‖ Each 2 liters of Medium G.S. contained 1600 cc. of 10 per cent aqueous yeast-water extract.

fluence of only those factors which affected the weight of mycelium. The outstanding factor is the glucose concentration of the medium; there is a progressive increase in the yield of pad with an

increase in glucose concentration. However, as will be shown later, the sterol content of the pad decreased. When the medium contained 2 per cent glucose, the yield of pad was about 7 gm. per pan; when 4 per cent was used the pad weighed 9 gm.; with 6 per cent it was 17 gm.; and with 8 to 10 per cent the weight equalled 29 gm. In four of the five concentrations of glucose, the yields of pad per square inch were greater in the case of the pans constructed with slightly raised covers than they were in the pans without raised covers. Evidently an increased air supply favored mycelium formation. As might be expected, the yield of pad is affected by the period of incubation. The pads obtained at the end of 9 to 11 days growth were tough and thick; those harvested at the end of 47 days incubation were highly sporulated, thin, and fragile. Supplementing the inorganic medium with organic material in the form of an aqueous extract of malt sprouts or yeast did not markedly increase the yield. Temperature exerted a slight effect, but not enough data are at hand from which to draw conclusions.

Sterol Determination—In the present experiments a colorimetric and a gravimetric method were studied as to their applicability for determining the sterol content of molds and mushrooms. The colorimetric procedure used was that of Myers and Wardell (13), but certain yellow and yellow-brown off colors present in the finally developed color forced us to abandon this method and to use the longer but more reliable gravimetric procedure.

In applying the gravimetric method (14) to fungus material, various steps in the procedure were studied, such as time of extraction, concentration of alkali, and time for hydrolysis of the combined sterols, extraction of the sterols from the hydrolyzed mixtures, and the effect of different procedures on the stability of the alcohol-soluble sterols. Each step was checked by recovery experiments with added ergosterol. If a recovery of 90 per cent or better of the added ergosterol was not obtained, the procedure was modified until this figure was reached. After many experiments, the details of which cannot be given here, the following procedure was adopted.

Final Procedure

Method of Extraction—20 gm. of fungus material, dried over P_2O_5 for 18 hours, were extracted with absolute alcohol for 3 hours in an

all glass Soxhlet extractor on an electric hot plate. The receiver was then changed and the extraction continued for another 3 hours with a fresh portion of alcohol. The combined extracts and rinsings were filtered before being made up to a volume of 300 cc.

Free Sterol Determination—Two 20 cc. aliquots in 50 cc. beakers, covered with watch-glasses, were heated to boiling on an electric hot plate. To each of the hot solutions 4 cc. of a 1 per cent solution of digitonin in 95 per cent alcohol were added. This was a sufficient excess of digitonin to cause complete precipitation of the digitonide as recommended by Thaysen (15) and Dam (16). After cooling, distilled water was added to the solutions until a cloudiness was produced. The beakers and contents were then placed in an ice box and the digitonides allowed to precipitate over a period of at least 15 hours, usually overnight. To prevent any loss of digitonide by solubility in alcohol, as pointed out by Fraser and Gardner (17), the contents of the beakers were evaporated practically to dryness before a fan at 45°. The residues were treated with small quantities of ether and washed by decantation. This was found to remove practically all of the colored impurities, leaving almost colorless digitonides. The latter, after having been broken up by means of a stirring rod, were transferred to weighed asbestos Gooch crucibles, where they were further washed with distilled water until no more foam or bubbles appeared in the wash water. The digitonides were dried for 2 hours in an electric oven at 105° and then weighed. The weights of the digitonides obtained from the aliquots of the different fungi varied from 8 to 26 mg.

Total Sterol Determination—In determining the total sterol, two 50 cc. aliquots of the alcohol extract were placed in 100 cc. Erlenmeyer flasks, 5 gm. of powdered KOH were added, and the mixtures saponified for $\frac{1}{2}$ hour under a reflux condenser in a boiling water bath. In the same manner, two 50 cc. aliquots were saponified with 0.5 gm. of KOH. Before extracting the sterols from the saponification mixtures with ether, most of the alcohol (35 cc.) was distilled off in a vacuum at 45°. The residual saponification mixtures were then diluted with water, and shaken out with three successive 35 cc. portions of ether in a separatory funnel. The combined ether extracts were washed with two portions of water (20 cc. each), and then made up to a volume of 100 cc. Two 50 cc. aliquots were taken for analysis. The ether, in 50 cc. beakers, was

TABLE III
Sterol Content of Various Fungi (Dry Basis)

Fungus	Glucose content of medium	Period of incu-bation	Alcohol-soluble sterol			Sterol in alcohol-ex-tracted residue
			Free	Total		
				1 per cent KOH	10 per cent KOH	
	per cent	days	per cent	per cent	per cent	per cent
Aspergillus niger, Culture 1........	4	7–12	0.39	0.40	0.39	0.14
" " " 1........	10	10	0.30	0.27	0.23	0.08
" " " 1........	20	10	0.09	0.22	0.21	0.06
Aspergillus oryzæ, Culture 1........	4	10	0.76	0.73	0.54	0.26
" " " 1........	10	10	0.23	0.28	0.29	0.01
" " " 1........	20	10	0.22	0.27	0.31	0.03
Aspergillus oryzæ, Culture 2........	4	10	0.63	0.51	0.50	0.09
" " " 2........	4	47–51	1.07	1.01	0.93	0.40
" " " 2........	4*	10	0.71	0.85	0.76	0.11
" " " 2........	4†	10	0.69	0.73	0.73	0.18
" " " 2........	4‡	10	0.58	0.53	0.52	0.07
" " " 2........	10	10	0.47	0.42	0.37	0.13
" " " 2........	20	10	0.19	0.21	0.20	0.02
Aspergillus oryzæ, Culture 192......	4	10	0.54	0.67	0.72	0.05
" " " 965......	4	10	0.98	0.96	0.99	0.15
" " " 965......	10	10	0.20	0.23	0.26	0.05
" " " 965......	20	10	0.31	0.34	0.34	0.09
Boletus clintonianus...............	Nature		0.28	0.47	0.30	0.04
Clitocybe multiceps................	"		0.33	0.43	0.37	0.04
" " (mycelium)....	4	10	0.50	0.53	0.52	0.09
Fusarium sp., Culture 20..........	4	9–13	0.35	0.43	0.33	0.05
Marasmius oreades................	Nature		0.37	0.28	0.24	0.03
Penicillium expansum.............	4	10	0.39	0.35	0.38	0.14
" "	10	10	0.20	0.27	0.25	0.07
" "	20	10	0.08	0.17	0.16	0.05
" janthinellum...........	4	10	0.16	0.13	0.14	0.06
Rhizopus nigricans................	4	14	0.18	0.13	0.08	0.04
Secotium acuminatum..............	Nature		0.10	0.24	0.20	0.10
Trichoderma sp., Culture W.......	4	10	0.20	0.28	0.23	0.08

* Each 2 liters of Medium G.S. contained 500 cc. of 10 per cent aqueous malt sprouts extract.

† Each 2 liters of Medium G.S. contained 1000 cc. of 10 per cent aqueous malt sprouts extract.

‡ Each 2 liters of Medium G.S. contained 1600 cc. of 10 per cent aqueous yeast-water extract.

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evaporated at 45° before a fan.   The residues were dissolved in 25 cc. of alcohol, and the total sterols determined as described for the free sterol determination.   The weights of the digitonides obtained were of about the same magnitude as those of the free sterol.   Multiplying the weight of the digitonides by 0.2412 gave the weight of sterol, calculated as ergosterol.

Alcohol-Insoluble Sterol—All of the above determinations were made on the alcohol extract of the fungus material.   In order to determine the sterols left unextracted by the alcohol, the fungus residue was saponified with 100 cc. of a 10 per cent alcoholic KOH solution for 1 hour under a reflux condenser in a boiling water bath.   The residue after saponification was filtered off and washed with alcohol until the washings were colorless.   After removal of most of the alcohol in a vacuum, the material was transferred to a separatory funnel with an equal volume of water and shaken with three portions of ether.   The washed ether extracts were made up to 100 cc. and 20 cc. aliquots were taken for analysis.   These were treated in the same manner as the aliquots taken for the total sterol determination.

Alkali-Insoluble Residue—In an earlier experiment on *Aspergillus oryzæ*, Culture 2, the fungus residue after alcohol extraction and saponification with 20 per cent KOH was refluxed with 5 per cent HCl for $\frac{1}{2}$ hour in a water bath, but no additional sterol could be detected even colorimetrically in the resulting extract.   Either there were no sterols present in the pad after alkaline saponification, or else, if some did remain, they were destroyed by the acid treatment.

Sterol Content of Molds and Mushrooms

Table III shows the sterol content of various fungi, determined according to the scheme of analysis described above.   The results indicate the following.

1. The sterol content of the fungi varies with the species. Considering only the molds grown under identical conditions (on Medium G.S. containing 4 per cent glucose, for a period of 10 days), we see that *Aspergillus oryzæ*, Culture 965 contains the largest quantity of free sterol, 0.98 per cent, and *Penicillium janthinellum* the smallest, 0.16 per cent.   The two common molds, *Aspergillus niger*, Culture 1 and *Penicillium expansum* contain about 0.4 per cent of free sterol.

2. The sterol extracted by alcohol is practically all present in the free state, because the values obtained after saponification with either 1 or 10 per cent alcoholic KOH are of the same order of magnitude as the free sterol figures.   Since the sterols are present in the free state, there is a possibility that some of them may be destroyed by saponification.   This might explain the fact that some of the total sterol values are less than the free, and some of the values obtained on saponification with 10 per cent KOH are lower than those obtained with 1 per cent KOH.   Dam has recently shown (18) that saponification in the presence of air, as was done in these experiments, tends to cause a loss of at least 1 to 5 per cent in recovery of cholesterol.

3. The sterol extracted by the alcohol represents 70 to 90 per cent of the total present in the fungus pad.   About 10 to 30 per cent additional sterol is obtained by treating the alcohol-extracted residue with 10 per cent alcoholic KOH.   This additional sterol is probably present in the fungus material in a conjugated form. As was mentioned before, treatment of the residue from the alkali saponification with 5 per cent HCl yielded no additional sterol.

4. Four mushrooms, gathered in the open, contained about the same percentage of sterol as some of the molds.

5. The sterol content varied not only with the species, but also with different strains of the same species.   The four strains of *Aspergillus oryzæ*, Cultures 1, 2, 192, and 965, grown under the same conditions, contained 0.76, 0.63, 0.54, and 0.98 per cent of sterol respectively.

6. Varying the period of incubation affected the sterol content of the mold pad.   For example, *Aspergillus oryzæ*, Culture 2 incubated 47 to 51 days contained 1.07 per cent of free sterol as compared to 0.63 per cent when grown for 10 days.

7. As might be expected, varying the composition of the medium changed the sterol content of the mycelium.   *Aspergillus oryzæ*, Culture 2, grown on the inorganic medium, contained about 0.6 per cent of free sterol.   When grown on this medium supplemented with an aqueous extract of malt sprouts it contained 0.7 per cent sterol.   Addition of yeast water to the inorganic medium did not produce an increase in the sterol content of *Aspergillus oryzæ*, Culture 2.   Increase of the glucose content of the medium caused a decrease in the sterol content of the mold pad.   On a 4 per cent

TABLE IV

Yield of Pad and Sterol Production per Pan of Various Molds Grown under Different Conditions

Mold	Period of incubation	Glucose content of medium	Weight of dry pad		Sterol	
			Per pan	Per 100 gm. of glucose	Dry pad	Per pan
	days	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*
Aspergillus niger, Culture 1...........	7–12	4	20.5	25.6	0.53	0.109
" " " 1...........	10	10	41.5	20.8	0.38	0.158
" " " 1...........	10	20	77.0	19.3	0.15	0.116
Aspergillus oryzæ, Culture 1...........	10	4	7.6	9.5	1.02	0.077
" " " 1...........	10	10	16.0	8.0	0.24	0.038
" " " 1...........	10	20	14.3	3.6	0.25	0.036
Aspergillus oryzæ, Culture 2..........	10	4	11.9	14.9	0.70	0.083
" " " 2..........	47–51	4	5.2	6.5	1.47	0.076
" " " 2..........	10	4*'	14.5	18.1	0.88	0.128
" " " 2..........	10	4†	17.5	21.9	0.87	0.152
" " " 2..........	10	4‡	15.2	19.0	0.65	0.099
" " " 2..........	10	10	29.1	14.6	0.60	0.175
" " " 2..........	10	20	25.0	6.3	0.21	0.053
Aspergillus oryzæ, Culture 965........	10	4	12.5	15.6	1.13	0.141
" " " 965........	10	10	26.3	13.2	0.25	0.066
" " " 965........	10	20	26.0	6.5	0.40	0.144
Penicillium expansum.................	10	4	18.3	22.9	0.53	0.097
" "	10	10	19.3	9.7	0.27	0.052
" "	10	20	36.0	9.0	0.13	0.047

* Each 2 liters of Medium G.S. contained 500 cc. of 10 per cent aqueous malt sprouts extract.

† Each 2 liters of Medium G.S. contained 1000 cc. of 10 per cent aqueous malt sprouts extract.

‡ Each 2 liters of Medium G.S. contained 1600 cc. of 10 per cent aqueous yeast-water extract.

glucose medium *Aspergillus oryzæ,* Culture 2 produced 0.63 per cent of free sterol; when the medium contained 10 per cent of glucose, the sterol content of the pad decreased to 0.47 per cent; and on a 20 per cent glucose medium the pad contained only about 0.20 per cent of sterol.

Similarly the sterol contents of *Aspergillus niger,* Culture 1 and *Penicillium expansum* decreased as the glucose content of the medium was increased from 4 per cent to 10 per cent or to 20 per cent. In the case of *Aspergillus oryzæ,* Culture 1 and *Aspergillus oryzæ,* Culture 965, although there was a distinct decrease in the sterol figures on a medium containing 10 per cent glucose as compared to 4 per cent, there was no marked difference between the effect of 10 per cent and that of 20 per cent glucose media.

In Table IV are shown the results of an attempt to determine under what conditions of growth the largest amounts of mycelium and sterol are produced. From Table III the percentages of sterol (free sterol plus sterol in alcohol-extracted residue) were obtained and used to calculate the weight of sterol per pan. It is evident that the molds differ in their utilization of glucose for mycelium formation. *Aspergillus oryzæ,* Culture 1 produced only 9.5 gm. of pad from 100 gm. of glucose, whereas *Aspergillus niger,* Culture 1 formed almost 3 times as much mycelium, 25.6 gm. *Penicillium expansum* also produced a large weight of pad, 22.9 gm. *Aspergillus oryzæ,* Culture 965 formed about the same weight of mycelium as *Aspergillus oryzæ,* Culture 2 on all three glucose concentrations. When *Aspergillus oryzæ,* Culture 2 was grown on a 10 per cent glucose medium, it produced about the same weight of mycelium per 100 gm. of glucose as when it was grown on a 4 per cent glucose medium. Approximately the same is true of *Aspergillus oryzæ,* Culture 1 and *Aspergillus oryzæ,* Culture 965. *Aspergillus oryzæ,* Culture 2 formed 20 to 50 per cent more mycelium, when grown on Medium G.S. supplemented with malt sprout extract and yeast water than it did when grown on Medium G.S. alone.

In the production of sterol either in the laboratory or on a commercial scale, the production per unit of apparatus is the important consideration. Gauged from this point of view, the yield of sterol ranged from 0.036 to 0.175 gm. per pan. The yield varied with (*a*) the species and strain, (*b*) the time of incubation, and (*c*) the composition of the medium.

Factors Affecting the Synthesis of Sterols—The conditions which play an important rôle in the synthesis of sterols by molds are as yet not definitely known. Belin (19) and Terroine and Bonnet (20) have shown that the total fatty acid content of *Sterigmatocystis nigra*, grown on Czapek's solution, can be increased by increasing the carbohydrate content of the medium. The latter investigators also showed that by increasing the carbohydrate content of the medium from 3 to 10 per cent the unsaponifiable matter increased from 0.3 to 1.0 per cent. Increasing the glucose content of the medium to 20 and 30 per cent did not further increase the quantity of unsaponifiable matter. Heiduschka and Lindner (6), in their studies on the ergosterol content of yeasts, have shown that the factors which are known to increase the fat content of yeast also increase the sterol content. Top beer yeast grown on malt-wort frequently saturated with oxygen contained 70 per cent more ergosterol than that grown on the normal wort solution. Addition of secondary sodium phosphate as well as alcohol to the beer-wort caused an increase in the ergosterol content of the yeast. The addition of both peptone and sodium phosphate resulted in a yield of ergosterol not quite equal to normal. Terroine and his coworkers (21), on the contrary, found no parallelism to exist between the content of fatty acid and that of sterol in either *Sterigmatocystis nigra* or in fatty seeds, and concluded that sterols are formed at the expense of fatty material and not that the sterols are produced simultaneously with fats in an interdependent process.

A possible mode of formation of ergosterol in the vegetable kingdom has been suggested by Heilbron and Sexton (22). Quoting these authors, "The common and probably general association of dihydrositosterol with sitosterol in vegetable oils suggests its genesis by a reduction process. Similarly, as there is every reason to believe that ergosterol is also present in all phytosterols, we venture to suggest with all reserve that concurrent with its reduction to dihydrositosterol (sitostanol), oxidation of sitosterol (possibly γ- sitosterol) to ergosterol occurs."

Although the factors and mechanism involved in the production of sterols by molds are by no means clearly known, certain relationships are emerging, and with further biochemical studies these new relationships will probably be more clearly established. The

subject is a field in which there will undoubtedly be increasing activity in the near future.

SUMMARY

The growth and sterol production of a number of molds under various conditions have been studied. In a preliminary survey, 55 molds were used to inoculate a synthetic, inorganic medium containing 4 per cent glucose as the source of carbon. Of these molds, twenty-three showed good growth in flasks. The yields of dry pad varied from 7.3 to 35.0 per cent of the glucose consumed.

Eleven of the twenty-three molds, representing the principal species, were grown in mass cultures in large tin pans. The weight of dry pad ranged from 9.5 to 25.6 per cent of the glucose utilized. Different strains of the same species also differed in their utilization of glucose. Increasing the glucose content of the medium to 10 or 20 per cent, in most cases, more than doubled the yield of dry pad. When the inorganic medium was supplemented with an aqueous extract of malt sprouts or fresh yeast, the mycelial growth of one of the strains studied, *Aspergillus oryzæ*, Culture 2, was increased from 20 to 50 per cent. Increasing the period of incubation of this strain from 10 to 50 days caused a 56 per cent decrease in the weight of pad.

The autoclaved, dried, and finely ground pads of twelve of the twenty-three molds showing good growth were irradiated with ultra-violet light and fed to rachitic rats to test their antirachitic potency. Five kinds of mushrooms, gathered in the open, were similarly tested for their antirachitic activitability. Eight of the molds and three of the mushrooms brought about distinct healing of rickets even when fed at a level of 10 mg. of irradiated material per rat over a period of 7 days.

As determined by the gravimetric digitonide method, the total alcohol-soluble sterol in eleven molds and four mushrooms varied from about 0.1 to 1.0 per cent of the dry weight of the fungus material. The amount of sterol left unextracted by the alcohol ranged from 0.01 to 0.40 per cent of the dry pad. In most cases, 90 per cent or more of the alcohol-soluble sterol was present in the free state.

The sterol content of the molds varied not only with the species, but also with different strains of the same species. Increasing the

glucose content of the medium caused a decrease in the percentage of sterol. Supplementing the inorganic medium with organic nutrients (aqueous extract of malt sprouts or fresh yeast) produced no appreciable change in the sterol content. Lengthening the period of incubation increased the percentage of sterol; the weight of pad, however, decreased so that the quantity of sterol per unit of apparatus remained about the same.

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ON THE FUNCTION OF HEXURONIC ACID IN THE
RESPIRATION OF THE CABBAGE LEAF

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It has been shown (3) in a previous paper (1928) that plants
which contain a peroxidase system and the suprarenal cortex of
animals contain a relatively high concentration of a substance
called hexuronic acid. This substance is characterized by its
high reducing power and its reversible oxidizability. It has been
suggested that hexuronic acid is involved as a catalyst in the
respiration of the cell. The subject of this paper is the inquiry
into the part this substance plays in the respiration of the cabbage
leaf.

If a part of the leaf is placed in the respirometer[1] in the presence
of potassium hydroxide, it shows a vigorous oxygen uptake of
between 2.5 and 6 c.mm. for each gm. of tissue each minute.
This rate of oxygen uptake remains constant for more than
an hour.

If, however, the leaves are ground in a meat mincer, which
injures most of the cells and releases the intracellular fluid, and the
pulp is placed in the respirometer, it shows a small oxygen uptake,
between 1 and 2 c.mm. for each gm. each minute. This shows
that mincing the leaves greatly injures the normal mechanism of
respiration.

In experiments in the respirometer, the first 5 minutes are lost
for observation because of the time necessary for the respirometer
to reach equilibrium as well as the few minutes required to weigh
the pulp and fill the respirometer. If at the beginning of the
readings a sample is taken, it will be found to contain practically
no reduced hexuronic acid. Since the acid is present in the
intact leaf chiefly in the reduced condition, it is evident that the

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[1] The thin marginal parts of inner leaves were used for these experiments.

reduced form disappears between the time of mincing and the beginning of the readings.

The disappearance of the reduced hexuronic acid can be followed by color reactions. 15 cc. of the Folin phenol reagent are placed in a series of beakers. The leaves are minced and the pulp quickly spread on a glass plate. At intervals of 2 minutes, samples of about 2.5 gm. of the pulp are placed in the reagent. After the first eight samples have been taken, the content of the beakers is filtered through muslin into test-tubes. The reduced hexuronic acid reduces the reagent without addition of alkali.

In the sample taken immediately after mincing, a deep blue color indicates the presence of a high concentration of reduced hexuronic acid. In the sample taken 2 minutes later, the color is moderately deep. In the third sample the color is rather faint. The fourth and fifth samples indicate only a trace; the remainder are negative.

This experiment shows that the relatively large amount of reduced hexuronic acid in the intact leaf disappears within the first 5 minutes after mincing. Since this disappearance does not take place if the pulp is kept in a vacuum, it is evident that the disappearance of the substance is due to its oxidation. During this interval, the pulp thus takes up oxygen at a rate of about the same order as that of the normal respiration. The absorption of oxygen lasts as long as reduced hexuronic acid is present. The pulp does not reduce oxidized hexuronic acid, or does so to a small extent only and indicates that the mechanism which brings about the reduction of hexuronic acid is damaged by the process of mincing to a greater extent than is the mechanism of the oxidation of the acid.

If the original concentration of reduced hexuronic acid is restored to the pulp by addition of this substance, a vigorous uptake of oxygen again takes place which lasts until the theoretic quantity of oxygen is taken up which is required for the reversible oxidation of the added acid. Fig. 1 shows the result of such an experiment.

Hexoxidase

If the pulp is quickly boiled, and then cooled, it will not be able to oxidize hexuronic acid. This shows that the oxidation of the acid in the fresh pulp is due to the presence of a thermola-

bile catalyst. For the sake of brevity this catalyst will be referred to as hexoxidase.

The first question to be studied was whether the hexoxidase is bound to the formed elements of the cell or is, in part at least, also present in the cell sap in solution. It was found that the hexoxidase is present to a large extent in the juice.

If the pulp is pressed through muslin, placed in the respirometer, and hexuronic acid is added, a great increase of oxidation shows the presence of the hexoxidase. The oxygen uptake, on
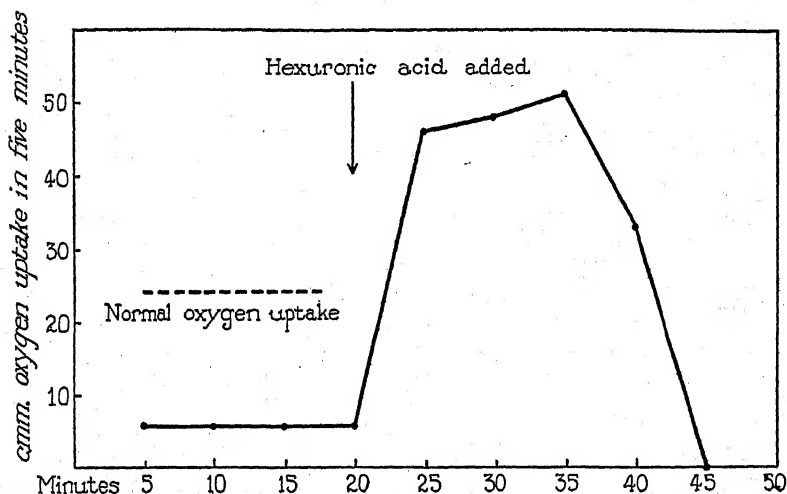
FIG. 1. The effect of hexuronic acid on the uptake of oxygen by minced cabbage leaves.

addition of the acid, will be the same if the juice is freed from all suspended material by centrifugation.

In Fig. 2 are shown the results of an experiment in which the pulp was pressed out at once and placed quickly in the respirometer.

If barium acetate is added to the juice a heavy precipitate is produced which can be separated in the centrifuge. The supernatant fluid shows undiminished activity.

If an equal quantity of saturated solution of ammonium sulfate is added to the juice, about half of the hexoxidase is precipitated.

If the juice is saturated with ammonium sulfate, all of the

hexoxidase will be found in the precipitate, which can be easily separated on the Buchner funnel. If the precipitate is dissolved in water or a phosphate buffer, it can be reprecipitated repeatedly practically without loss of activity. Such a preparation obtained by precipitation with ammonium sulfate does not show spontaneous uptake of oxygen in the respirometer.

If the ammonium sulfate precipitate is suspended in M/15 phosphate solution of pH 5.9 and is allowed to stand overnight in the ice box, an inactive precipitate will settle, which can be
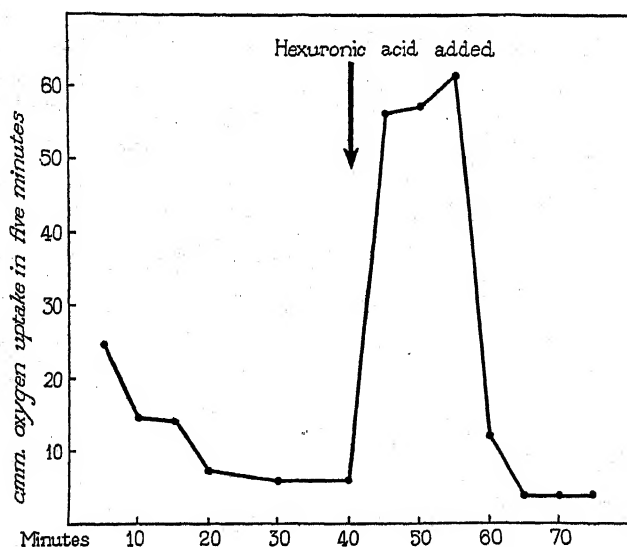
Fig. 2. The effect of hexuronic acid on the uptake of oxygen by juice pressed from cabbage leaves.

separated in the centrifuge, leaving a limpid, highly active enzyme solution.

All further observation will deal with the enzyme which had been precipitated from a solution saturated with ammonium sulfate and redissolved in M/15 phosphate buffer of pH 5.9 (corresponding to the pH of the cabbage juice).

The hexoxidase is resistant to many different chemical and physical agents. The preparations can be kept for approximately a week in the ice box without loss of activity. The wet ammonium

sulfate precipitate can be dried in the air or in a vacuum. The solution can be frozen in solid carbon dioxide. A solution of 1 per cent acetic acid, or of sodium carbonate or formalin, will not destroy its activity in 5 minutes. The dried preparation can be extracted with ethyl ether without loss of activity or by absolute alcohol or alcohol-ether with but little loss of activity.

Rapid heating to boiling will completely destroy the enzyme. Solutions of the enzyme or the wet ammonium sulfate precipitates are very sensitive to solvents like alcohol or acetone. Treatment with three times its own volume of methyl alcohol destroys 80 per cent of the activity. Acetone destroys it almost completely.

The enzyme is not sensitive to the cyanide ion, showing that in its action a mechanism usually classified as "oxygen activation" is not involved. Sodium cyanide 0.005 per cent has no effect on the rate of oxidation; 0.01 per cent has a small inhibitory action. High concentration, as 0.1 per cent, inhibits oxidation to a great extent. It has been shown by Dixon (1927) (1) and by me (1926) (2) that such a high concentration of cyanide inhibits enzymes in a non-specific way. Shardinger's enzyme is inhibited by such high concentration, although oxygen activation is not involved in its action. As emphasized by Dixon (1927) (1) only the inhibition by small concentrations of cyanide is characteristic for oxygen activation.

As criterion for hydrogen activation the reduction of methylene blue is usually applied. It is easy to demonstrate that the reduction of methylene blue by hexuronic acid is not enhanced by the presence of hexoxidase. Also narcotics, such as 5 per cent of urethane or chloroform, have no inhibitory influence.

The insensitivity of the enzyme to cyanide gives further evidence of the significance of this enzyme, together with hexuronic acid, in the normal respiration of the cabbage leaf. It was found that the respiration of intact leaves is inhibited only to 6 per cent by the presence of 0.01 per cent of sodium cyanide. The respiration of thin slices of a plant containing a phenol-oxidase, the potato, was found to be inhibited in an identical experiment up to 60 or 75 per cent by the same concentration of cyanide.

The results show the mechanism of activation of hexoxidase to be different from that of all other oxidizing enzymes. So do the kinetics of its activity. If the rate of oxidation in varying con-

centrations of hexuronic acid is plotted, a straight line is obtained, which slopes but gently, and the rate of oxidation tends to become parallel to the abscissa in low concentrations of the acid. The results of two of the several experiments performed are shown in Fig. 3. The same result can also be obtained by a different method. If hexuronic acid is mixed with the hexoxidase and samples are taken at regular intervals and the quantity of the unoxidized acid is plotted against time, an almost straight line is
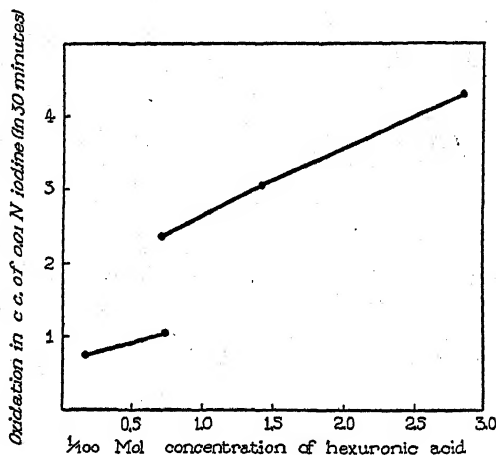
FIG. 3. The effect of concentration on the velocity of oxidation of hexuronic acid by hexoxidase.

obtained, until oxidation of the acid is almost complete. The experiments were carried out at room temperature in beakers with continuous mechanical shaking. The quantity of unoxidized hexuronic acid was followed iodometrically.

No other substance has been found besides hexuronic acid on which the hexoxidase will act. Pyrogallol, catechol, quinol p-phenylenediamine, the diamine plus naphthol, leucoindophenol, all remain unoxidized.[2]

[2] It should be mentioned that the pulp of cabbage leaves or the pressed juice oxidizes dibrom-indophenol white at a high rate. This reaction is sensitive to cyanide. By precipitation with ammonium sulfate the juice loses this activity.

Also glutathione[3] remains unoxidized in the presence of hex-oxidase.   If, however, hexuronic acid is present, the glutathione is oxidized; the hexuronic acid plays the rôle of catalyst; it is oxidized by the enzyme and reduced by glutathione.[4]

. A study of the velocity of oxidation of hexuronic acid shows that the oxidizing enzyme is of a complex nature.   The probable mechanism by which the enzyme brings about its effect has been discussed in a recent publication (4).   The curves which represent the velocity of oxidation of hexuronic acid in the presence of the enzyme support the hypothesis that an intermediate substance, x, is involved.   If the rate of oxidation of hexuronic acid is rapid compared to the rate of oxidation of x, the line in Fig. 3 would be parallel to the abscissa.   The gentle slope indicates that the rate of oxidation of x to the rate of oxidation of hexuronic acid by x stands as 1 to 3.

<div align="center">EXPERIMENTAL</div>

Experiment 1.   Uptake of Oxygen by Minced Cabbage Leaves— The outer leaves of the cabbage are rejected.   The thick main ribs of the inner leaves are cut out with a sharp knife, and the leaves are quickly minced in a meat mincer, which reduces them to a relatively fine, juice pulp.   A sample of 813 mg. is quickly weighed and transferred to a respirometer of the Warburg type and suspended there in 2 cc. of a $M/15$ phosphate buffer of pH 5.9.

The side arm of the respirometer is filled with 0.5 cc. of a solution of hexuronic acid, brought to pH 5.9 with disodium phosphate. This sample reduces 3.4 cc. of 0.01 N iodine and requires thus for its reversible oxidation about 200 c. mm. of oxygen.

0.4 cc. of potassium hydroxide solution is placed in the central tube of the respirometer to absorb carbon dioxide.   The respirom-eter kept at 24° is closed and the readings which are begun after 5 minutes, are repeated at intervals of 5 minutes.

[3] Crystalline glutathione was prepared by the method of Kendall.

[4] Formaldehyde in the presence of the enzyme preparation leads to rapid disappearance of hexuronic acid (iodometrically) and of cysteine and glutathione.   This is, however, not an oxidative process since it occurs anaerobically at the same rate.   Acetaldehyde is only slightly active.   Propionic aldehyde, heptylic aldehyde, crotonic aldehyde, ben-zaldehyde, cinnamic aldehyde, anisic aldehyde, vanillin, and dimethyl *p*-aminobenzaldehyde are inactive.

After four readings, the side arms are dumped. Since the side arms cannot be washed out, the dumping is not quantitative. The horizontal dotted line in the curve (Fig. 1) gives the oxygen uptake of the same weight of intact cabbage leaves.

Experiment 2. Uptake of Oxygen by Pressed Cabbage Leaves— Cabbage leaves are minced quickly. The pulp is wrapped in muslin, pressed by hand, and 2 cc. of the juice pipetted into the respirometer. Further details are the same as those given in Experiment 1. The curve obtained is shown in Fig. 2.

*Experiment 3. Effect of Concentration on the Velocity of Oxidation—*A 0.01 M solution of hexuronic acid is prepared by the addition of 8.9 mg. of hexuronic acid to a solution of hexoxidase in 5 cc. of phosphate buffer pH 5.9. The solution is gently shaken for 30 minutes and is then acidified with acetic acid and titrated with a solution of 0.01 N iodine. Similar experiments were made with solutions which contained 0.015 M, and with 0.03 M, concentrations of hexuronic acid. The influence of the concentration of hexuronic acid is shown in Fig. 3.

Preparation of Hexuronic Acid

The hexuronic acid used in the experiments was prepared in the chemical laboratory of The Mayo Foundation from suprarenal glands of beef. Since (for the purpose of constitutional analysis) greater amounts of hexuronic acid had to be prepared, the methods of preparation described elsewhere (1928) were applied for quantities of 25 kilos of glands. It was found, however, that the original method was inadequate for work on such a large scale. The original method was thus modified in several respects. By this modified procedure, I have obtained 4 gm. of crystalline hexuronic acid from 25 kilos of glands. The same yield has been obtained by my assistant, Miss Bair. More than 20 gm. of crystalline hexuronic acid have been prepared in this way.

The steps of the preparation were the following. The glands were cut from the freshly killed animals at the Swift packing plant at South St. Paul, Minnesota. They were at once trimmed, and packed in solid carbon dioxide, in which they were transferred to the clinic, where they were minced still in frozen condition. Then to every kilo of the pulp, 1 liter of 5 per cent trichloroacetic acid was added, and the whole mass was thoroughly mixed.

After half an hour, the fluid was separated in the Sharples centrifuge. 40 gm. of neutral lead acetate for each kilo of glands were dissolved in a small quantity of hot water. To every liter of the solutions of the glands 0.1 cc. of 20 per cent sodium cyanide was added. Two-thirds of the lead acetate solution was added to the solution; then a 10 N solution of sodium hydroxide was added with rapid agitation until the fluid distinctly turned the indicator brom-thymol blue. The rest of the lead acetate was then poured in. The mixture was cooled with ice for half an hour; the precipitate was separated in the Sharples centrifuge.

The precipitate was suspended in about 2 liters of water and 25 per cent sulfuric acid was added, until the fluid was distinctly acid to brom-thymol blue. Then the maximal amount of 20 per cent phosphotungstic acid was added which just did not leave any excess of this acid in the solution after being cooled with ice. (The necessary quantity was estimated on a small sample, sodium hydrosulfite being used as indicator for free phosphotungstic acid after the precipitate had been separated.) The solution was filtered on a suction filter.

The further steps of the method were similar to those described in the previous paper (1928).

SUMMARY

Evidence is given that hexuronic acid plays an important part in the respiration of the cabbage leaf. It connects, as hydrogen carrier, the system in which the molecular oxygen enters into reaction with the system which supplies hydrogen and is involved in the oxidation of the foodstuff. There is in the cabbage leaf a highly active enzyme which in the presence of oxygen rapidly oxidizes hexuronic acid to its reversible oxidation product. The name hexoxidase is used for this enzyme, the properties of which are described.

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X-RAY ANALYSIS OF BONE AND TEETH

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PLATES 1 AND 2

INTRODUCTION

The chemical composition of bone has been intensively studied
by a succession of investigators dating from Hoppe-Seyler and
lately exhaustively studied by Kramer and his associates (6, 7).
Exact knowledge of the chemical composition of bone is necessary
for an intelligent study of whether or not equilibrium exists
between bone and body fluids. To know simply that Ca, CO_3,
and PO_4 exist in bone, however, is not sufficient as previous
experiments by one of us have illustrated (Hastings, Murray,
and Sendroy (3)). Equilibration of blood serum with $CaCO_3$
(calcite) led one group of investigators to the conclusion that
serum might be in equilibrium with at least one bone constituent,
$CaCO_3$; equilibration of serum with $Ca_3(PO_4)_2$ led another group
(4) to conclude that serum was supersaturated with respect to
$Ca_3(PO_4)_2$; and equilibration of serum with $CaHPO_4$ has resulted
in a third group (6) concluding that serum was undersaturated
with respect to $CaHPO_4$. All of these conclusions may be
independently correct and yet have no relation to the situation
existing between serum and bone. It becomes of paramount
importance to know whether or not the calcium salts of bone
exist in crystalline form and what this crystal form is.

An attack on this problem has been made by de Jong (5) who
concluded that a mineral was present in bone belonging to the
apatite series and which can be represented by the formula

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$3Ca_3(PO_4)_2 \cdot CaCO_3$. Gassmann (1) has presented chemical evidence that the composition of bones and teeth may be represented by the formula

$$\left[Ca \begin{pmatrix} OPO_3 & Ca \\ >Ca & \\ OPO_3 & Ca \end{pmatrix}_3 CO_3 \right]$$
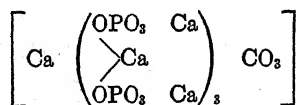
While our work was in progress Taylor and Sheard (8) published a study of the refractive index and x-ray spectrograms of bone, teeth, various pathological concretions, and certain calcium salts. They concluded that bone contains a mineral of the apatite series having the type formula $3Ca_3(PO_4)_2 \cdot CaX_2$ but does not contain the mineral brushite, $CaHPO_4 \cdot 2H_2O$ in significant amount.

This paper contains the results of a study of bone and various calcium salts by means of x-ray spectrograms. In the main, our results confirm those of de Jong (5), and of Taylor and Sheard (8).

The questions which we attempted to answer were: (1) Does bone contain definite crystalline salts? (2) With what mineral does bone correspond? (3) Is $CaHPO_4$ present in bone? (4) Is the substance known chemically as tertiary calcium phosphate, $Ca_3(PO_4)_2$, crystalline and is it present in bone? (5) What rôle does calcium carbonate play in the structure of bones? (6) Does the crystalline character of teeth differ from that of bone? (7) What is the size of the unit crystal cell?

Techniques and Methods

Powder Photographs—The x-ray spectrograms were secured by passing a beam of monochromatic x-rays through a mount on which were placed the two samples, so that a comparison might be made.

The samples were prepared by powdering finely in an agate mortar till the powder would pass through a 100-mesh sieve. The powdered samples were then placed in a mount which consisted of a piece of x-ray film from which the emulsion had been removed. The mount had the dimensions of approximately 2 cm. long, 0.5 cm. wide, and 0.025 cm. thick. Two rectangular holes were cut opposite each other in which the samples were

placed. The mount was then enclosed with cigarette paper. The complete mount was then placed in position at the center of a cassette which formed a quadrant of a circle, and the film was placed on the circumference.

Monochromatic x-rays from a molybdenum target filtered by a zirconium filter were used. The incident wave-length was 0.712 Å. The x-rays were defined by a narrow slit, and the exposure was approximately 45 hours.

The resulting photograph contained the x-ray spectrograms of the two substances to be compared. The distance of each line from the zero line was measured in cm. and tabulated. Also the spacings of the spectral lines in Ångström units were recorded with a General Electric ruler.

Laue Photograph—A beam of x-rays was passed through a section of the enamel of teeth. The sections were cut from the enamel normal to the axis of growth, and normal to the vertical surface. These sections were mounted on a glass slide over a small hole bored in the glass, so that the beam of x-rays would pass through only the section of the tooth. The section was then ground to a thickness of approximately 0.40 cm., the grinding being done on a ground glass surface with wet emery.

The sections were subjected to both beams of white radiation and monochromatic x-rays. The white radiation was secured from a tungsten Coolidge tube of universal type, operated at approximately 71,000 peak volts. The time of exposure was approximately 20 hours. Data from the photographs so secured were not valuable for computations but they did show the presence of crystal planes in the enamel, and furnished information concerning their orientation.

Monochromatic x-rays were secured from the same machine from which the powder photographs were made. The rings secured by passing a beam of monochromatic x-rays through a section of enamel gave definite information concerning the symmetry of the crystals inside the enamel.

Results

An example of an x-ray spectrogram of bone and the mineral dahlite is given in Fig. 1. The bone used in this experiment was a specimen of young bone supplied by Dr. Charles B. Hug-

gins. It had been prepared by his method osteogenesis and was not more than 30 days old. It is apparent by inspection that a close similarity exists between lines present in the bone spectrogram and the dahlite spectrogram. Spectrograms of the tooth enamel and of a sample of tertiary calcium phosphate are also included in Fig. 1.

A more exact comparison may be made, however, by plotting the data obtained from such films (Tables I to III) in the manner shown in Text-fig. 1. Here the spacings between the planes are plotted in Ångström units along the abscissæ and the estimated intensity of the lines, referred to the strongest line as 100, are plotted as ordinates. The first point of significance is that bone presents characteristic and reproducible x-ray spectrograms. This may be interpreted as indicating that bone is built up of minute crystals which are oriented at random. The second point of interest is that the strongest line of the bone, corresponding to a spacing of 2.72 Ångström units, is present in old and young bone, whether fresh or ashed, in enamel or dentine, in fluoroapatite, chloroapatite, the carbonate apatite (dahlite), and in the substance known as tertiary calcium phosphate. The third point of significance is the fact that most of the lines found in an x-ray spectrogram of bone and enamel are found in the spectrograms of the mineral dahlite and correspond as to spacings and approximately as to intensities. The fourth point of significance is that the prominent lines characteristic of secondary calcium phosphate are absent from the spectrograms of bone and enamel. For example, the strongest line found in the spectrogram of $CaHPO_4$ corresponding to a spacing of 2.91 Ångström units, is entirely absent from the spectrogram of bone. By mixing bone and $CaHPO_4$ in the ratio 9 : 1 characteristic lines of $CaHPO_4$ were visible. It may therefore be stated that $CaHPO_4$, if present, is there in an amount less than 10 per cent of the total weight. It is perhaps of importance that young bone, prepared by Huggins' technique, the oldest portion of which was not more than 30 days old yielded a spectrogram in which no $CaHPO_4$ lines were present. It is of further importance that no lines corresponding to those found in the form of calcium carbonate, known as calcite, are present in the bone spectrograms. These two points make it apparent that studies such as one of us has

TABLE I

Comparison of Spacings in Ångström Units Obtained from x-Ray Spectrograms of Bone, Teeth, and Various Apatites

Fluoro-apatite	Chloro-apatite	Dah-lite	Tertiary calcium phosphate	Enamel	Dentine	Bone	Ossified dog bone	Ether extract bone cortex	Bone cortex ashed at high temperature
									4.05
3.85			3.85	3.85					3.85
	3.81								
		3.75							
	3.35	3.35	3.35	3.35	3.35	3.35	3.35	3.35	
3.40									3.40
3.15	3.15								
									3.20
3.02	3.05	3.02	3.02	3.02	3.02	3.02	3.02	3.02	3.05
									2.80
2.72	2.72	2.72	2.72	2.72	2.72	2.72	2.72	2.72	2.72
2.60	2.60	2.60	2.60	2.60					2.60
2.51									2.50
2.25	2.26	2.24	2.25	2.25	2.25	2.25	2.25	2.25	2.25
2.13	2.13	2.12	2.13	2.13					2.13
2.05	2.02		2.05	2.05					2.02
1.93	1.93	1.93	1.93	1.93	1.93	1.93	1.93	1.93	1.93
1.88	1.88	1.87	1.88	1.89					1.88
1.83	1.82	1.83	1.83	1.83	1.83	1.83	1.83	1.83	1.83
1.79									1.78
1.75	1.75		1.75						1.75
1.71	1.71	1.71	1.71	1.71	1.71	1.71	1.71	1.71	1.71
1.64	1.64	1.62		1.64					1.64
1.53	1.52	1.51	1.53	1.53					1.53
1.49	1.49	1.49	1.49	1.49					1.49
1.44	1.44	1.44	1.44	1.44	1.43	1.44	1.44	1.44	1.44
1.34	1.32	1.33		1.34					1.34
1.305		1.30	1.305	1.305		1.305			1.30
1.27	1.27	1.27	1.27	1.27					1.27
1.25		1.25							1.25
1.23	1.23	1.23	1.23					1.23	1.23
1.21		1.20		1.21	1.21	1.21			1.21
1.14	1.14	1.14	1.14	1.14	1.14		1.14	1.14	1.14
1.10	1.10	1.11	1.10	1.10	1.10	1.10	1.10	1.10	1.10
1.09		1.09							
1.07		1.07	1.06						
1.02	1.03	1.02	1.02	1.02	1.02	1.02	1.02	1.02	1.02
0.98									
0.97	0.97	0.98	0.98	0.98	0.93				0.97

A few lines of shorter spacing have been omitted in this table.

TABLE II

Comparison of Intensities in Per Cent Obtained from x-Ray Spectrograms of Bone, Teeth, and Various Apatites

Fluoro-apatite	Chloro-apatite	Dah-lite	Tertiary calcium phosphate	Enamel	Dentine	Bone	Ossified dog bone	Ether extract bone cortex	Bone cortex ashed at high temperature
									5
2			5	5				15	
	5								
		10							
	25	20	25	25	5	20	25	20	
15									
5		15							60
									20
5	15	15	10	10	2	5	10	10	20
									85
100	100	100	100	100	100	100	100	100	100
20	15	10	20	20					75
2									5
25	40	20	10	10	3	25	20	20	50
20	15	5	5	5					50
20	20		25	25					20
40	50	25	10	10	5	20	25	25	15
20	20	15	5	20					75
40	50	25	20	40	5	20	25	25	40
5									75
5	20		2						30
15	10	5	15	20	5	20	25	25	40
10	5	5		10					20
15	5	2	15	5					20
10	20	2	2	2					20
15	40	10	15	20	3	10	15	15	25
2	10	2		2					5
10		5	5	5		2			10
10	15	2	5	2					15
5		2							15
5	35	5	5	10	2	5		5	20
5		2							10
5	10	5	5	5	2		5	5	10
5	20	5	5	5	2	5	5	5	20
5	3								
2		1	2						5
2	5	3	2	2		2	5	5	
2									
2	10	3	2	2					2

TABLE III

Comparison of Spacings and Intensities of Bone, CaHPO₄, and Calcium Carbonates

Line No.	Bone		CaHPO₄		Calcite		Aragonite		μ—CaCO₃*	
	Spacing	Intensity	Spacing	Intensity	Spacing	Intensity	Spacing	Intensity	Spacing	Intensity
	Å.		Å.		Å.		Å.		Å.	
1	3.35	20	3.30	95	3.75	60	3.80	5	3.59	75
2	3.02	5	3.10	20	3.39	20	3.25	100	3.29	75
3	2.72	100	2.91	100	2.98	100	2.67	25	2.71	60
4	2.25	25	2.71	90	2.72	15	2.45	15	2.31	30
5	1.93	20	2.60	5	2.45	50	2.32	40	2.05	100
6	1.83	20	2.45	15	2.25	50	2.16	10	1.85	25
7	1.71	20	2.21	10	2.07	50	2.10	10	1.82	25
8	1.44	10	2.14	5	1.90	75	1.96	40	1.64	50
9	1.30	2	2.06	5	1.85	75	1.86	40	1.53	5
10	1.21	5	1.97	5	1.59	45	1.80	30	1.46	5
11	1.10	5	1.90	20	1.50	45	1.72	40	1.36	5
12	1.02	2	1.84	25	1.46	20	1.61	5	1.28	30
13			1.78	15	1.41	30	1.52	10	1.14	5
14			1.71	35	1.33	5	1.49	5	1.10	5
15			1.67	20	1.28	20	1.46	10		
16			1.63	15	1.23	20	1.40	15		
17			1.60	5	1.17	35	1.35	20		
18			1.56	5	1.14	40	1.26	10		
19			1.53	5	1.05	2	1.23	35		
20			1.50	5	1.03	40	1.20	20		
21			1.47	5	1.01	35	1.18	5		
22			1.44	5	0.98	30	1.16	15		
23			1.40	2	0.96	30	1.12	10		
24			1.35	20	0.93	30	1.10	5		
25			1.31	5	0.89	10	1.08	5		
26			1.26	5	0.85	5	1.04	20		
27			1.22	5	0.81	5	1.02	20		
28			1.20	10	0.79	5	1.00	20		
29			1.18	10	0.78	2	0.97	15		
30			1.14	5	0.77	2	0.95	5		
31			1.11	5	0.70	2	0.93	5		
32			1.09	5	0.68	2	0.90	2		

* These data are taken from the work of Gibson, Wychoff, and Merwin (2).
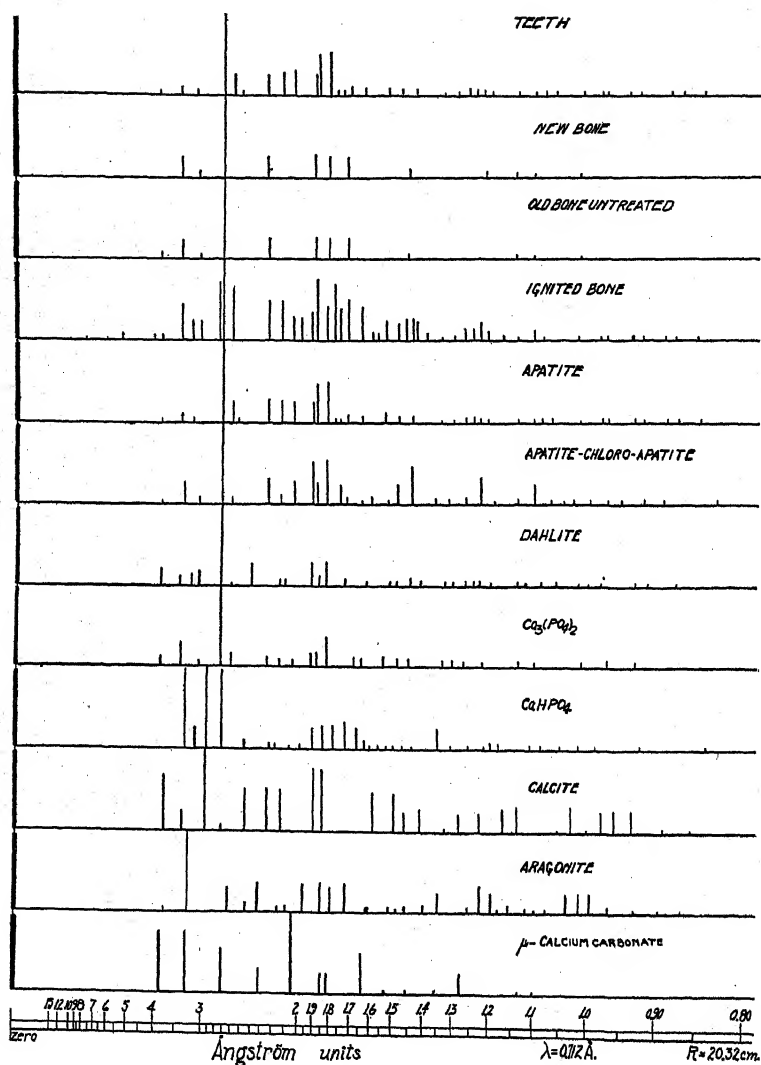
TEXT-FIG. 1.   The data obtained from x-ray spectrograms plotted with intensities as ordinates and spacings as abscissæ.

made on equilibria between biological fluids and calcite, $CaCO_3$, or such as others have made on equilibria between biological fluids and secondary calcium phosphate, $CaHPO_4$, are without biological significance.

It would appear that such equilibrium experiments should be carried on between biological fluids and dahlite if the conclusions to be drawn are to be capable of biological interpretation.

Regarding the question of the independent existence of the chemical substance $Ca_3(PO_4)_2$ it may be stated that it may exist
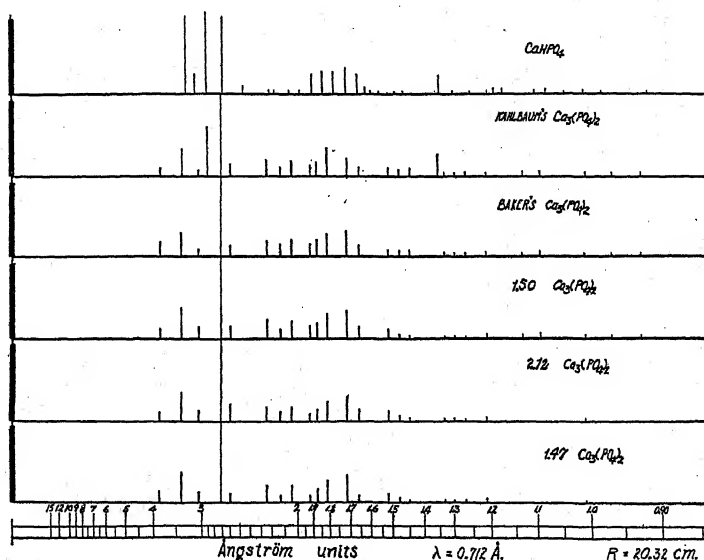
TEXT-FIG. 2.   The data obtained from x-ray spectrograms plotted with intensities as ordinates and spacings as abscissæ.

as a definite independent crystal form and further appears to be the fundamental nucleus of the members of the apatite series. It may have associated with it CaO, $CaCl_2$, CaF_2, $CaCO_3$, etc. so placed within the crystal unit that no difference in the important planes is detectable.   An analogy to such a situation exists in the case of amphibole, a member of the asbestos family, in which there may be substitution of a variety of the acidic and basic atoms without detectable difference in the spectrograms

(Warren (9)). Our conclusion therefore is that a substance of the chemical analysis $Ca_3 (PO_4)_2$ may exist, that it belongs to the apatite series of mineral, but that it probably does not represent as stable a form of the series as is represented by the general form $CaX_2 \cdot nCa_3(PO_4)_2$ where X may be Cl, F, $\frac{1}{2}$ O, or $\frac{1}{2}CO_3$, and n is not less than 2 or greater than 3.

Through the kindness of Dr. L. Emmet Holt we were supplied with a variety of specimens of tertiary calcium phosphate which varied in analysis both below and above the theoretical Ca :P ratio of 1.50. x-Ray spectrograms of these salts were taken and the data of three have been plotted in Text-fig. 2 together with those obtained from spectrograms of two commercial samples of tertiary calcium phosphate. The data of the spectrogram of $CaHPO_4$ have been added for reference. It will be seen that the spectrograms of all of the specimens of tertiary calcium phosphate are essentially identical except in the case of the Kahlbaum salt. In this sample the 2.91 and the 1.35 lines, characteristic of $CaHPO_4$, were present. This is interpreted as indicating the presence of $CaHPO_4$ in this salt.

Conclusions Concerning the Crystal Cell

The Laue photographs furnished interesting information concerning the structure of bone and enamel. An example of a Laue photograph secured with white radiation is shown in Fig. 2. Concentric rings were secured for sections cut from any portion of the enamel. The rings were very narrow and defined in the case of monochromatic radiation. The fact that concentric rings were secured makes it evident that the crystals of the enamel have no definite orientation in regard to histological elements, the so called rods and prisms. The crystals are packed in the rods and prisms at random and covered with a sheath which composes the histological unit.

It was interesting to study the possible crystal structure of the unit crystal. With the postulate that the enamel crystal was similar to that of apatite, due to the similarity of patterns and intensity of the lines in the spectrograms, an effort was made to account for all the reflecting planes. All the lines were

accounted for on the basis of a close packed hexagonal lattice containing 4 molecules which when referred to orthorhombic axes gave the constants: $a_0 = 20.8 \times 10^{-8}$ cm., $b_0 = 12.0 \times 10^{-8}$ cm., $c_0 = 8.82 \times 10^{-8}$ cm. The degree of correspondence

TABLE IV

Comparison of Theoretical and Observed Spacings of the Unit Crystal Cell of Enamel

Line No.	Intensity	Distance	Radians	Degrees	Sine θ	Observed d_{gij}	GE scale	Theoretical d_{gij}	Plane
		cm.				Å.	Å.	Å.	
1	2	3.32	0.0811	4° 38′	0.0808	4.41	4.40	4.41	002
2	5	3.75	0.0916	5° 15′	0.0915	3.89	3.85	3.92	130
3	25	4.30	0.1051	6° 1′	0.1048	3.39	3.35	3.36	402
4	10	4.79	0.1170	6° 41′	0.1164	3.06	3.02	3.02	620
5	100	5.32	0.1299	7° 26′	0.1294	2.75	2.73	2.75	531
6	20	5.55	0.1356	7° 44′	0.1346	2.64	2.60	2.60	800
7	10	6.46	0.1577	9° 2′	0.1570	2.26	2.25	2.25	802
8	5	6.80	0.1661	9° 31′	0.1653	2.15	2.13	2.18	842
9	25	7.10	0.1734	9° 56′	0.1725	2.06	2.05	2.04	404
10	20	7.55	0.1844	10° 34′	0.1834	1.94	1.93	1.96	260
11	20	7.73	0.1889	10° 41′	0.1854	1.92	1.89	1.92	134
12	40	7.95	0.1942	11° 7′	0.1928	1.84	1.83	1.80	524
13	20	8.52	0.2081	11° 55′	0.2065	1.72	1.71	1.73	660
14	10	8.89	0.2172	12° 26′	0.2153	1.65	1.64	1.66	844
15	5	9.50	0.2320	13° 17′	0.2298	1.55	1.53	1.55	372
16	2	9.80	0.2395	13° 43′	0.2371	1.50	1.49	1.50	080
17	20	10.14	0.2477	14° 11′	0.2450	1.45	1.44	1.44	480
18	2	10.95	0.2674	15° 19′	0.2641	1.34	1.34	1.36	664
19	5	11.20	0.2736	15° 40′	0.2700	1.32	1.305	1.32	046
20	2	11.50	0.2807	16° 4′	0.2768	1.28	1.27	1.28	806
21	10	12.00	0.2931	16° 47′	0.2888	1.23	1.21	1.21	484
22	5	12.85	0.3137	17° 57′	0.3082	1.15	1.15	1.15	302
23	5	13.35	0.3261	18° 41′	0.3203	1.11	1.10	1.11	702
24	2	14.50	0.3541	20° 18′	0.3469	1.02	1.02	1.02	448
25	2	15.20	0.3713	21° 17′	0.3630	0.98	0.98	0.98	520
26	2	15.95	0.3896	22° 18′	0.3795	0.94	0.93	0.93	668

between the theoretical and observed spacings is shown in the seventh and ninth columns of Table IV. This correspondence would lead to the conclusion that the possible crystal structure of enamel is hexagonal.

SUMMARY

1. x-Ray spectrograms of bone indicate that it has a crystalline structure.

2. Estimations of the spacing between the planes and the intensities of the lines indicate that bone has a crystal structure fundamentally the same as that of other members of the apatite series.

3. Since chemical analysis indicates that the chemical composition of untreated bone is similar to that of the mineral dahlite and since x-ray spectrograms indicate the similarity in the crystal structure of bone enamel and dahlite, it is concluded that the calcium salts of bone and enamel may be represented by the formula: $CaCO_3 \cdot nCa_3(PO_4)_2$, where n is not less than 2 nor greater than 3.

4. No evidence is found that $CaHPO_4$ or $CaCO_3$ exists in bone or teeth as such.

5. $Ca_3(PO_4)$ is crystalline and seems to belong to the apatite series.

6. The unit crystal cells of enamel have a random distribution irrespective of the histological elements, the so called rods and prisms.

7. The diffraction lines can possibly be accounted for on the basis of a close packed hexagonal lattice which when referred to orthorhombic axes has the lattice constants: $a_0 = 20.8 \times 10^{-8}$ cm., $b_0 = 12.0 \times 10^{-8}$ cm., $c_0 = 8.82 \times 10^{-8}$ cm.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. A reproduction of x-ray spectrograms of (1) tooth enamel, (2) dahlite, (3) ashed bone, (4) tertiary calcium phosphate.

PLATE 2

FIG. 2. A Laue photograph of tooth enamel.
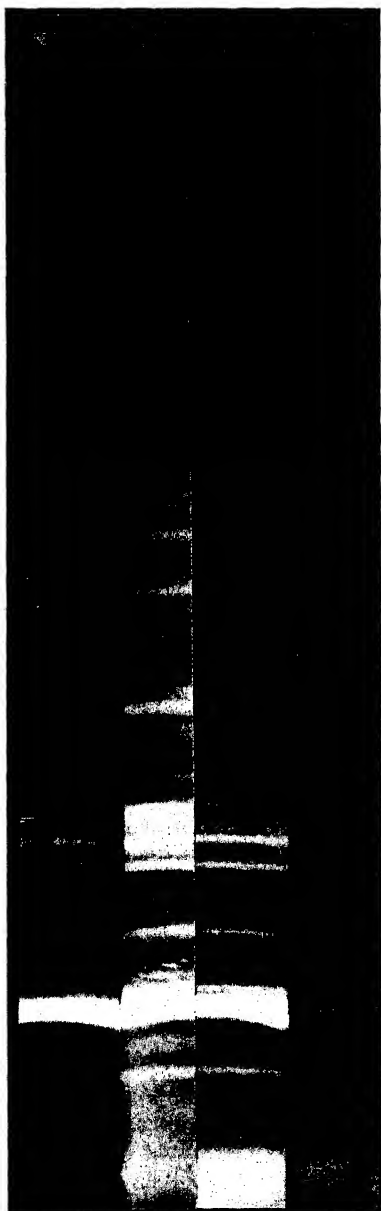
FIG. 1

(Roseberry, Hastings, and Morse: x-Ray analysis of bone and teeth)

Fig. 2

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A STUDY OF GLUTATHIONE

VI. THE PREPARATION OF OXIDIZED GLUTATHIONE

By HAROLD L. MASON

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About a year ago Hopkins (2) published the results of his re-investigation of glutathione. He described the preparation of oxidized glutathione by aeration of the pure tripeptide at pH 7.6 and showed that the product contained less nitrogen and sulfur and more carbon and hydrogen than the theoretical values. These findings led Hopkins to the conclusion that during the oxidation there was some destruction of the glutathione, possibly by the hydrogen peroxide formed, with consequent loss of nitrogen as ammonia and of sulfur as sulfuric acid or in a volatile form. The apparent loss of nitrogen and sulfur was cited as an illustration of the instability of the tripeptide when pure. This paper will present evidence to show that nitrogen and sulfur are not lost during aeration, that the glutathione molecule remains intact during the process, and that the low values obtained for nitrogen and sulfur are caused by the presence of alcohol in the preparations. The only instability shown in aqueous solution up to a temperature of 62° is the spontaneous loss of the glutamyl radical as pyrrolidonecarboxylic acid as described in the preceding paper of this series (5). At higher temperatures more deep-seated changes occur as was shown by Hopkins who first isolated pyrrolidonecarboxylic acid after glutathione had been boiled with water. A detectable loss of nitrogen and sulfur from glutathione has never been observed except when it was treated with relatively strong alkali or when boiled with water. The evidence indicates that under physiologic conditions glutathione is no more unstable than other peptides with the one exception just mentioned.

Oxidized glutathione was prepared by aeration of the tripeptide in slightly alkaline solution and the product was isolated by pouring its concentrated solution into absolute alcohol.   Analysis of the product gave results which were similar to those of Hopkins. Ferricyanide and iodine were also used to oxidize the tripeptide with the same result, that is, the products isolated contained about 12 per cent less nitrogen and sulfur than the calculated values.

It was observed that in the case of ferricyanide and iodine (in the presence of excess iodide) the amount of oxidant used was just sufficient to convert all of the sulfhydryl content to disulfide. Meldrum and Dixon (6) have shown the same thing to be true for oxygen.   It is therefore difficult to account for loss of nitrogen and sulfur by oxidation.   Since Hopkins has shown that no further loss of nitrogen or sulfur occurs when oxidized glutathione is aerated at pH 7.6 an assumption that these elements are lost after oxidation is untenable.   A careful search for ammonia and hydrogen sulfide during aeration and in the aerated solution did not reveal a trace of either.   Furthermore, examination of the analytical data reveals the fact that Hopkins' preparations and those described in this paper contain nitrogen and sulfur in the ratio of 3 atoms of nitrogen to 1 atom of sulfur.   This is a strong indication that the glutathione molecule is intact.   If 1 atom of sulfur were lost it would necessitate the simultaneous loss of 3 atoms of nitrogen.   The only logical possibility which remains is that the oxidized glutathione has not lost sulfur and nitrogen but has taken up an impurity which contains carbon and hydrogen but no sulfur or nitrogen.   In consideration of the method of preparation this could only be alcohol.   Such a conclusion was at first rejected on account of the care taken to remove alcohol from the preparations, but experiment soon showed the correctness of this conclusion.

Preparation 9 which had been dried to constant weight at 80° in the vacuum produced by an efficient water pump was tested for alcohol as follows: A solution of 80 mg. in 8 cc. of water was distilled.   The distillate (3 cc.) gave a positive iodoform test.   All the other preparations which were tested likewise gave a positive test for alcohol.

Another preparation (No. 1) which had been dried at 100° and then *in vacuo* at room temperature was submitted to various tests and analyses.   After a positive iodoform test a quantitative esti-

mation showed the presence of 12 per cent of alcohol. At a temperature of 111° and in a high vacuum the material lost 11.8 per cent of its weight. An elementary analysis agreed very well with the values calculated on the assumption that 1 molecule of oxidized glutathione is associated with 2 molecules of alcohol. Titration of the material with alkali according to the method of Harris (1) showed a ratio of carboxyl groups to amino groups of 2:1 and an equivalent weight of 180. The equivalent weight calculated for $GSSG \cdot 2C_2H_6O$ is 176. The material which had been heated at 111° *in vacuo* was also titrated with alkali. Although the ratio of COOH to NH_2 was still 2:1, the total amount of alkali used was only three-fifths of the amount calculated for GSSG.

A third preparation (No. 6) which had been dried *in vacuo* over sulfuric acid at room temperature and then found to contain 88 per cent of the theoretical amount of nitrogen and sulfur lost 13.6 per cent of its weight at 111° in a high vacuum. An elementary analysis after heating gave figures which were not far from the values calculated for GSSG, although the carbon and hydrogen were a little high and the nitrogen and sulfur a little low. The loss in weight was somewhat more than the calculated value. A carboxyl titration showed a ratio of COOH to NH_2 of 2:1 but the total carboxyl groups found were only two-thirds of the calculated amount.

The analytical results indicate that 1 molecule of oxidized glutathione holds approximately 2 molecules of alcohol. This proportion probably varies somewhat depending on the conditions which attend the treatment with alcohol. My preparations possessed nitrogen and sulfur contents which varied from 88 to 94 per cent of the calculated values for oxidized glutathione. The alcohol is held with remarkable tenacity. Under the conditions mentioned 28 to 48 hours were required for the attainment of a constant weight.

The problem of the preparation of pure oxidized glutathione then resolves itself into the elimination of alcohol from the product as usually prepared or the use of some other means of dehydration. It was thought that the alcohol could be removed by the use of a temperature of 110–120° and a high vacuum. The loss in weight of the two preparations so treated and the elementary analysis of

one of them supported this idea, but the carboxyl titrations showed that some change occurred which reduced the number of carboxyl and amino groups.   The explanation is not obvious since assumptions as to anhydride formation do not agree with the loss in weight and the elementary analysis.   Two carboxyl groups have disappeared for each amino group.   If the presence of alcohol is not objectionable no attempt need be made to eliminate it since the nitrogen content may be safely taken as a measure of the amount of oxidized glutathione present.

Although it cannot be stated with certainty that Hopkins' preparations of oxidized glutathione contained alcohol, that is the most probable explanation since he used absolute alcohol to dehydrate them.   A comparison of Hopkins' data with the analysis of one of my preparations shows the similarity.

	C	H	N	S	N:S
Hopkins............................	39.51	6.36	12.07	9.52	2.90
Hopkins............................	39.89	6.98	11.99	9.60	2.85
Preparation 1 (H. L. M.)..........	40.21	6.31	12.10	9.29	2.98
Theoretical........................	39.21	5.27	13.72	10.46	3.00

The high per cent of carbon and hydrogen indicates the presence of alcohol.   Loss of NH_3 and SH would lower the hydrogen content since they would be replaced by OH or O.   Since Hopkins evaporated his preparations to dryness, only volatile products could have been lost.   The extra hydrogen in the solid material must be due to alcohol unless a deep-seated decomposition of the glutathione has occurred with the possible loss of carbon dioxide in addition to sulfur and nitrogen.   Since such an assumption is entirely contrary to the evidence presented, it is impossible to escape the conclusion that oxidized glutathione precipitated or dehydrated with alcohol retains some alcohol in spite of the use of means which are usually effective for its removal.

EXPERIMENTAL

Preparation of Oxidized Glutathione

1. Oxygen as Oxidant—Following the previous descriptions of Hopkins and others, 1 to 2 gm. of crystalline glutathione were

dissolved in 25 cc. of water, a trace of ferric sulfate was added, the solution was made just alkaline to litmus with barium hydroxide, and then aerated until the nitroprusside reaction was negative. The barium was exactly removed with sulfuric acid. The filtrate was evaporated to 4 to 5 cc. and poured into 100 to 150 cc. of absolute alcohol. When the precipitate was friable the mixture was filtered and the solid dried over sulfuric acid *in vacuo*.

2. Ferricyanide as Oxidant—The details of this procedure are not of practical significance since the preparation by aeration is much to be preferred. An outline will suffice to make the method

TABLE I

Nitrogen and Sulfur Content of Preparations of Oxidized Glutathione

Preparation No.	Oxidant	Nitrogen	Sulfur	N:S
		per cent	per cent	
1	Oxygen	12.10	9.29	2.98
2	Ferricyanide	12.56		
3	"	12.36		
4	"	12.89	9.85	3.02
5	Oxygen	12.16		
6	Ferricyanide	12.1	9.20	3.00
7	Iodine	12.8		
8	"	12.3		
9	Ferricyanide	11.5	9.01	2.92
10	Oxygen	12.2		
Theoretical values..............		13.72	10.46	

Preparations 1, 2, 8, 9, and 10 were tested for alcohol. The test was positive in all instances.

clear. The crystalline glutathione was oxidized by addition of ferricyanide to a solution kept slightly alkaline by frequent additions of barium hydroxide. For most of the preparations potassium ferricyanide was used. The solution was then acidified with sulfuric acid and ferrocyanide and excess ferricyanide was removed with ferric and ferrous sulfates respectively. The precipitate was centrifuged out and the solution concentrated under reduced pressure to less than 100 cc. Potassium sulfate was then removed by addition of alcohol until its concentration was 80 per cent. Most of the alcohol was removed from the filtrate

by evaporation under reduced pressure.   After addition of water, the excess sulfuric acid was removed with barium hydroxide.   The further procedure was that described in the preceding paragraph. In later work hydroferricyanic acid was used.   This was prepared just before use, by addition of the calculated amount of sulfuric acid to a solution of lead ferricyanide.

3. Iodine as Oxidant—I have shown previously that in the presence of sufficient iodide ion, iodine converts reduced gluta- thione practically quantitatively into the disulfide (4).   Accord- ingly, the oxidation was carried out by addition of a solution of iodine in hydriodic acid to a solution of the glutathione which also contained hydrogen iodide.   Iodide was then exactly removed with silver sulfate and sulfate with barium hydroxide.   The prod- uct was then isolated as described.   A summary of the various preparations is given in Table I.

Detailed Analysis of Preparation 1

1. Carbon and Hydrogen—Substance, 0.1040, 0.1102 gm.; CO_2, 0.1534, 0.1625 gm.; H_2O, 0.0589, 0.0620 gm.   Found: C, 40.22, 40.21; H, 6.33, 6.29.   Calculated for $C_{20}H_{32}O_{12}N_6S_2$.   C, 39.21; H, 5.27.   Calculated for $C_{20}H_{32}O_{12}N_6S_2 \cdot 2C_2H_6O$.   C, 40.88; H, 6.29.

2. Estimation of Alcohol—A solution of 0.0904 gm. in 50 cc. of water acidified with sulfuric acid was distilled.   About 35 cc. of distillate were caught in 25 cc. of 0.1 N permanganate acidified with 12 cc. of 5 N sulfuric acid.   The remainder of the procedure was carried out as directed by Kolthoff (3).   In this method the alcohol is oxidized to acetic acid.   9.43 cc. of 0.1 N permanganate were used.   Found alcohol, 12.0 per cent.   Calculated for GSSG·· $2C_2H_6O$.   Alcohol, 13.1 per cent.

3. Loss of Volatile Material—A sample of 0.4051 gm. was heated at the boiling point of toluene (111°) in the vacuum pro- duced by an efficient oil pump for 48 hours.   The loss of weight was very slow but ceased at the end of this time.   The loss was 0.0478 gm. or 11.8 per cent.

4. Carboxyl Titrations—The method used was that described by Harris.   The first part of the titration was performed in the ab- sence of formaldehyde with brom-thymol blue as the indicator. Then 10 cc. of neutral 40 per cent formalin were added and the titration again carried to the brom-thymol blue end-point.   The

preparation was titrated both before and after heating. The results are given in Table II.

TABLE II

Details of Titrations

Preparation 1.

Sample, gm.	0.0993, before heating	0.1016, after heating
	cc.	cc.
Total carboxyl found	$0.55 \times N$	$0.40 \times N$
Carboxyl groups found in absence of formaldehyde	$0.28 \times$ "	$0.21 \times$ "
Total amino groups found, by difference	$0.27 \times$ "	$0.19 \times$ "
" carboxyl groups calculated for $G_2S_2 \cdot 2C_2H_6O$	$0.56 \times$ "	$0.58 \times$ " *
Total amino groups calculated for $G_2S_2 \cdot 2C_2H_6O$	$0.28 \times$ "	$0.29 \times$ " *
Method sensitive to	$0.01 \times$ "	$0.01 \times$ "
Equivalent weight found	180	254
" " calculated	176	153

* Calculated for G_2S_2 these values are 0.66 cc. and 0.33 cc. of N alkali respectively.

TABLE III

Results of Carboxyl Titration after Heating

Preparation 6.

Sample, gm.	0.0935
	cc.
Total carboxyl found	$0.40 \times N$
Carboxyl groups found in absence of formaldehyde	$0.20 \times$ "
Total amino groups found by difference	$0.20 \times$ "
" carboxyl groups calculated for G_2S_2	$0.61 \times$ "
" amino groups calculated for G_2S_2	$0.305 \times$ "
Method sensitive to	$0.01 \times$ "

Detailed Analysis of Preparation 6

1. Loss of Volatile Material—In 28 hours at 111° and at a pressure less than 1 mm., 1.437 gm. lost 0.1960 gm. or 13.6 per cent. Calculated for $GSSG \cdot 2C_2H_6O$, 13.1 per cent.

2. Elementary Analysis after Heating—Substance, 0.1130, 0.0813 gm.; CO_2, 0.1648, 0.1185 gm.; H_2O, 0.0565, 0.0404 gm.   Substance, 0.1361, 0.1832 gm.; $BaSO_4$, 0.0998, 0.1391 gm.   Substance, 7.17 mg.; N (micro-Kjeldahl), 0.961 mg.   Calculated for $C_{20}H_{32}O_{12}N_6S_2$. C, 39.21; H, 5.27; N, 13.72; S, 10.46.   Found: C, 39.78, 39.80; H, 5.50, 5.57; N, 13.4; S, 10.07, 10.19.

3. Carboxyl Titration after Heating—The results are given in Table III.

SUMMARY

Evidence is presented to show that by aeration at pH 7.6 glutathione is quantitatively converted into the oxidized form without loss of nitrogen or sulfur.   The isolated preparation when dehydrated with alcohol contains approximately 2 molecules of alcohol for each molecule of the oxidized glutathione.   The alcohol is held with great tenacity and has not yet been successfully removed to yield a pure preparation.   However, the nitrogen content can safely be used as a measure of the amount of oxidized glutathione present.   Further work is in progress.

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STUDIES IN CARBOHYDRATE METABOLISM

II. GLUCOSE-LACTIC ACID CYCLE IN DIABETES*

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In the previous paper of this series (Himwich, Koskoff, and Nahum, 1929–30) a glucose-lactic acid cycle was demonstrated between muscle and liver of non-diabetic dogs. "Lactic acid liberated from muscle finds its way to the liver through the blood stream, and is there converted to carbohydrate. The liver in turn yields glucose to the blood, thus affording a supply of carbohydrate to muscle. . . . It must therefore become apparent that by virtue of this cycle, the liver becomes an integral part of the mechanism of recovery." That this cycle functions in the diabetic animal was anticipated from a study of the literature.

It is well known that anaerobic cleavages of carbohydrates are uninhibited in diabetes. With the onset of the diabetic state the liver rapidly becomes poor in glycogen which presumably leaves that organ in the blood of the hepatic vein in the form of glucose. The glycogen of muscle also decreases and may appear in the blood as lactic acid (Himwich, Loebel, and Barr, 1924; Loebel, Barr, Tolstoi, and Himwich, 1924; Doisy, Briggs, Weber, and Koechig, 1924–25; Hetzel and Long, 1925–26).

That portion of the cycle which takes place only at the expense of energy derived from oxidations, namely the production of carbohydrate from lactic acid, also occurs in diabetes, for Lusk and coworkers (1906, 1915, 1919, 1921), Embden and Salomon (1905), and Parnas and Baer (1912), have shown that lactic acid injected or fed to depancreatized or phlorhizinized animals is recovered in the urine as extra glucose. The present work was undertaken to

* A preliminary report of this work appeared in *Proc. Soc. Exp. Biol. and Med.*, **26**, 120 (1928–29).

determine the effect of the diabetic condition upon both phases of the glucose-lactic acid cycle.

Method

Nine dogs were studied on the 3rd or 4th day after pancreatectomy. Five other dogs were phlorhizinized in the manner described by Coolen (1895) and were examined after the D:N ratio had become constant. The remaining procedures employed were similar to those of the previous study (Himwich, Koskoff, and Nahum, 1929–30). After exposure of the vessels of the femoral triangle and a longitudinal abdominal incision, samples of blood were drawn from the femoral artery, femoral vein, portal vein, and hepatic vein in rest or during exercise produced by induction shocks applied to the lower extremities at the rate of one stimulus per second. Blood proteins were precipitated in the earlier experiments according to the procedure of Folin and Wu (1919) but in the later work the method of Somogyi (1930) was used. Glucose was estimated by the methods of Shaffer and Hartmann (1920–21) and Hagedorn and Jensen (1923). Then reducing substances were precipitated according to the procedures of Salkowski (1879) and Van Slyke (1917), and the lactic acid content of the filtrate was determined by the technique of Friedemann, Cotonio, and Shaffer (1927). As before, a difference of at least 5 mg. per cent of lactic acid and glucose was considered significant.

Results

The data of the depancreatized dogs are comprised of ten observations obtained during rest, eight in exercise, and four after work was completed. For economy of presentation all of the observations (twenty-two) will not be tabulated. However, the lactic acid and glucose results obtained in several representative experiments are given in Table I. The reciprocal effects of muscle and liver on the lactic acid of the blood may be observed during rest, exercise, and also following work, for muscle adds lactic acid to the blood while the liver removes that substance. The results for glucose are the reverse, since muscle removes hexose, while the liver liberates it.

The results of the lactic acid exchanges in the eight observations of the five phlorhizinized dogs were similar to those obtained on

the depancreatized animals although the differences in the phlor-hizinized dogs were less marked (Table II).   The findings regard-ing glucose exchanges were more definite.   In three of four ob-

TABLE I
Blood of Depancreatized Dogs

	Date	Hepatic vein	Portal vein	Femoral artery	Femoral vein	Remarks
	1928	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	
Lactic acid content	July 25	30	37	29	32	Rest
	" 10	60	80	75	82	65 min. exercise
	Aug. 2	32	66	38	44	80 " after work
Glucose content	July 25	322	294	324	310	Rest
	" 26	480	448	466	450	2 hrs. exercise
	Aug. 2	370	324	370	322	80 min. after work

TABLE II
Lactic Acid Content of Blood of Phlorhizinized Dogs

Date	Hepatic vein	Portal vein	Femoral artery	Femoral vein	Remarks
1928	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	
Mar. 8	22	29	34	35	Rest
Jan. 10	26	29	23	30	"

TABLE III
Glucose Content of Blood of Phlorhizinized Dogs

Date	Femoral artery	Femoral vein	Remarks
1928	*mg. per cent*	*mg. per cent*	
Nov. 28	58	67	Rest
	56	66	Exercise
	77	76	" 70 min.
	77	61	" 106 "

servations the liver added glucose and once the difference was within the experimental error.   The results for muscle are differ-ent from those usually observed, since glucose was added to the blood three times, while on four other occasions the muscle re-

moved glucose and once the difference was within the experimental error.   Table III presents the findings of an experiment where muscle surrendered glucose in the first two observations during rest and exercise.   On longer stimulation the level of the blood sugar rose so that finally after 106 minutes of exercise sugar passed from blood to muscle.   The results upon the blood drawn from the portal vein were varied.

In Table IV it may be seen that the liver glycogen decreased in two of four experiments where consecutive determinations were made.   In the other two experiments no changes were observed.

TABLE IV

Glycogen Content of Liver of Depancreatized Dogs

Date, *1923*	July 26	Aug. 2	Aug. 4	Aug. 16	July 10
	mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
Rest	0.226	0.089	0.082	0.304	0.088*
" 1 hr.			0.079		
" 2½ hrs.			0.081		
Work, 1 hr.		0.113			0.053†
" 2 hrs.	0.080				
Post-work, 1 hr.					0.028
" 2 hrs.				0.217	
" 4 "		0.095		0.174‡	

* Muscle.
† Muscle at 80 minutes.
‡ 3¾ hours.

DISCUSSION

On the basis of the ratios established by Burton-Opitz (1911) that 0.3 of the afferent hepatic blood supply is carried in the hepatic artery and 0.7 in the portal vein, it is possible to calculate the exchanges of glucose and lactic acid of the blood traversing the liver during rest.   The following values are the averages of the ten observations obtained from resting depancreatized dogs: the muscle added 8 (deviation of the mean, $A. D.^1 = 1.7$) mg. per

[1] $A. D. = \dfrac{a. d.}{\sqrt{n}}$ where $a. d.$ is the mean numerical deviation and n is the number of observations.

cent of lactic acid to the blood and removed 17 (*A. D.* 4) mg. per cent of glucose, while the liver absorbed 10 (*A. D.* 1.7) mg. per cent and liberated 23 (*A. D.* 4.8) mg. per cent.

	Muscle *mg. per cent*	Liver *mg. per cent*
Lactic acid.....................................	+8	−10
Glucose..	−17	+23

Before considering the significance of these results it may be well to emphasize the fact that the return flow from an organ consists not only of venous blood but also of lymph.   Hence less of any substance is removed from blood passing through an organ than is indicated by the differences between the content of the arterial and venous blood.   For the same reason more of the substance is added to blood than can be determined from the arterial-venous differences.   For example, Himwich and Castle (1927) have shown that the lymph flow was approximately 1 per cent of the blood draining the resting lower extremities, and on this basis the lactic acid liberated by muscle would be raised to 9 mg. per cent and the glucose removed would be diminished to 13 mg. per cent. From two observations (unpublished results) in which the oxygen capacity of the blood passing through the liver was determined, the lymph flow of that organ also was 1 per cent of its total minute volume, under these experimental conditions.   When the lymph flow is taken into consideration, the liver would be removing 10 mg. per cent of lactic acid and adding 27 mg. per cent of glucose. Therefore, the corrected results on the resting dogs are as follows:

	Muscle *mg. per cent*	Liver *mg. per cent*
Lactic acid.....................................	+9	−10
Glucose..	−13	+27

Since the rates of blood flow through muscle and liver are not the same, an erroneous impression is obtained when exchanges of glucose and lactic acid are considered in terms of 100 cc. of blood.   A true picture must present the exchanges occurring in a given time interval.   It can be calculated that 432 cc. of blood per minute passed through the resting musculature of the experimental animals since the blood flow is 9 cc. per 100 gm. per minute (Himwich and Castle, 1927) and the total musculature is approximately 4.8 kilos or 0.3 (Schleier, 1923; Junkersdorf, 1925) of the

average weight (16 kilos) of the dogs.   Therefore, 100 cc. of blood
require 0.23 minutes to pass through muscle.   In that time 77
cc. of blood traverse the liver, for its blood flow is 84 cc. per 100
gm. per minute (Burton-Opitz, 1911), and the average weight of
the organ in our experiments is about 400 gm. or 2.5 per cent of
16 kilos.   Hence, the values for the exchanges of liver should be
multiplied by 0.77 and the tabulation becomes

	Muscle	Liver
	mg. per cent	*mg. per cent*
Lactic acid	+9	−8
Glucose	−13	+21

This tabulation is quite instructive.   In the first place the liver
absorbs most of the lactic acid liberated by muscle.   Other or-
gans may also remove lactic acid (Himwich and Nahum, 1929).
In the second place, muscle takes only a portion (13 mg.) of the
glucose poured out by the liver (21 mg.).   The remainder (8
mg.) must be accounted for by that part of the glucose which is
eliminated by the kidney, as well as the portion removed by other
organs.   From a study of the urinary excretion of the experimen-
tal animals during the day previous to that on which the blood
samples were drawn, it appears that 5 mg. of glucose were excreted
in 0.23 minutes.   This accounts for all but 3 mg. per cent, an
amount within the experimental error.   However, other tissues
may also absorb glucose (Himwich and Nahum, 1929).

Thus far we have been considering the effects of the exchanges
of glucose and lactic acid in the various organs, chiefly muscle
and liver.   Additional information may be obtained from a study
of the glucose and lactic acid exchanges in the same organ.   For
example, what is the relationship between the lactic acid entering
the liver and the glucose leaving it?   The liver yields 21 mg. of
glucose and receives only 8 mg. of lactic acid.   Thus 13 mg. of
glucose or about 0.6 of the total must come from other sources.
It is conceivable that the additional glucose comes from fat,
but the weight of evidence is against this possibility (Lusk, 1928;
Rapport, 1930).

The breakdown of protein probably provides most of the 5 mg.
of the glucose excreted by the kidneys, for it is not likely that on
the day before the observations were made on the blood, approxi-
mately 3 days after pancreatectomy, the muscle was yielding any

considerable amount of extra glucose.   The origin of the remaining 8 mg. of glucose must be sought elsewhere.   An examination of Table IV reveals two instances when a definite decrease in the glycogen content of the liver occurred during the collection of the blood samples.   It is quite possible that an amount of glucose sufficient to explain the remainder of the extra glucose liberated by the liver came from the breakdown of the small quantity of glycogen still present in that organ.

The data obtained on muscle are more unequivocal.   Landsberg (1914) observed the removal of glucose by the perfused lower extremities of depancreatized dogs and concluded that the glucose removed was oxidized by muscle.   Macleod and Pearce (1914) and Mann and Magath (1923) have observed rapid decreases in blood sugar of depancreatized eviscerated dogs.   However, in view of the greatly diminished oxidations of carbohydrate, there are other interpretations more probable.   Certainly the glucose removed by the muscle of diabetic dogs is not stored as glycogen. Minkowski (1893) and Ringer, Dubin, and Frankel, (1921) have shown that muscles of depancreatized and phlorhizinized dogs contain but small quantities of glycogen.   Moreover, the glucose absorbed by muscle (13 mg. per cent), can be accounted for by the lactic acid liberated (9 mg. per cent), since these values are sufficiently close to be considered within the experimental error.

In phlorhizinized dogs a loss of glucose from muscle has been noted.   Although the experiments of Mann· and Magath (1923) and Soskin (1927) have demonstrated the inability of muscle glycogen to maintain the level of blood sugar it is possible that under the conditions of phlorhizinization, a small amount of glucose may appear from such a source.   With the glucose leaving the body through the kidneys, it is quite possible that at times the level of the glucose in the blood may be lower than that of the tissues and in such cases glucose may be liberated by muscle.

The slight differences both in lactic acid and glucose contents of phlorhizinized dogs are probably due to the small amount of carbohydrate present in the body.   Apparently at the time of the study the phlorhizinized dogs contained little carbohydrate.

Glycogenesis—It is probable that the lactic acid removed by liver is converted to carbohydrate even in total diabetes.   It has been shown that following injections of insulin there is an accumu-

lation of glycogen in the livers of depancreatized (Banting *et al.*, 1922) and phlorhizinized (Cori, 1925) animals. However, the presence of insulin may simply be necessary for the accumulation of glycogen. In diabetes the diminished glycogen content of the liver (Table IV) may be due to the fact that glycogenesis does not exceed glycogenolysis. Probably in the muscle, too, carbohydrate is formed from lactic acid. It is known for muscles *in situ* (Himwich and Castle, 1927; Himwich and Rose, 1929) as well as for excised muscle (Takane, 1926; Richardson, Shorr, and Loebel, 1930) that recovery may occur at the expense of fat, since the respiratory quotient varies within the physiological limits. In muscle excised from depancreatized dogs, Richardson, Shorr, and Loebel (1930) observed a respiratory quotient close to 0.7, that is, energy is furnished almost entirely by fat. In the present experiments the blood glucose removed by muscle was not necessarily oxidized, since, as shown above, it can be accounted for by glycolysis. We are therefore left with a picture of lactic acid transformed to carbohydrate by means of energy obtained from the oxidation of fat. At present we have no certain knowledge of the respiratory quotient of the liver; however, it may be expected that in that organ, too, fat furnished the energy for glycogenesis.

Taken as a whole, these results indicate that the same cycle which occurs in the normal dog also obtains in the diabetic animal. As in the normal dogs, muscle and liver play reciprocal rôles. Muscle absorbs glucose and liberates lactic acid. Although the kidney eliminates a portion of the lactic acid when it exceeds the threshold of 30 to 40 mg. per cent (Hewlett, Barnett, and Lewis, 1926–27), the liver continuously removes lactic acid and liberates glucose instead. In our dogs the excretion of lactic acid in the perspiration was probably negligible, although it has been found to be an important factor in human beings (Snapper and Grünbaum, 1929). These experiments, especially those on the depancreatized dogs with minimal amounts of glycogen in the liver and high level of glucose in the blood, bring out strikingly the point that lactic acid is a precursor of the glucose of the blood. This emphasizes the indirect method by which muscle glycogen may become available in the intact animal, a process which therefore cannot occur after hepatectomy.

SUMMARY AND CONCLUSION

Twenty-two observations have been made on nine depancreatized dogs, and eight observations on five phlorhizinized dogs. They all indicate, especially those on depancreatized dogs, that lactic acid is liberated by muscle and removed chiefly by liver, while glucose is poured out by liver to be absorbed by muscle and other tissues or excreted. Thus the glucose-lactic acid cycle between muscle and liver functions in diabetes just as it does under normal conditions. It is clearly demonstrated that by means of this mechanism, lactic acid, a waste product of muscular activity, is converted into a precursor of the glucose of the blood, accounting in the present experiments for approximately 0.4 of the glucose released by the liver.

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THE ACTION OF FORMALDEHYDE ON AMINO ACIDS WITH SPECIAL REFERENCE TO THE FORMA-TION OF AMINES*

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HISTORICAL

Effect of Formaldehyde on Protein Hydrolysis—In an effort to determine the exact nature of the formation of the humin fraction in protein hydrolysates, Gortner and Holm (1–3) made a study of the effects of the presence of various aldehydes on the hydrolysis of several proteins.

They found that when a protein is hydrolyzed in the presence of formaldehyde and the resulting hydrolysate analyzed by the Van Slyke method, the nitrogen distribution is so altered as to bear no resemblance to the analysis conducted in the absence of aldehyde. For proteins containing tyrosine and tryptophane hydrolyzed in the presence of increasing amounts of trioxymethyl-ene, both the soluble and insoluble humin fractions increase rapidly to a maximum after which there is a sharp drop. The ammonia fraction, on the other hand, decreases with the smaller additions of trioxymethylene and then rises rapidly for larger additions of the aldehyde. When neither tyrosine nor tryptophane is present in the protein, there is no change in either of the humin fractions with trioxymethylene, but there is a steady increase in the ammonia fraction.

Gortner and Holm believe that the sudden initial drop in the

ammonia fraction is due to the removal of tryptophane to the insoluble humin fraction, the tryptophane in the absence of aldehyde contributing to the ammonia fraction.

The increase in the so called "ammonia" fraction with larger additions of trioxymethylene was found to be due not to ammonia, but to volatile alkaline compounds which were not identified.

A preliminary study of this volatile nitrogen fraction was made by Gortner and Holm (2), who prepared the chloroplatinate of this excess ammonia fraction and found it to contain 3.52 per cent of nitrogen and 38.91 per cent of platinum. On the assumption that the formula of the chloroplatinate is represented by X_2PtCl_6, this platinum percentage would correspond to a molecular weight of 46 for X as contrasted with the expected value of 18 for (NH_4). Evidently, then, this second fraction was not ammonia, but was composed chiefly of other volatile basic nitrogen compounds.

Action of Formaldehyde on Ammonia—Aliphatic aldehydes in general react with ammonia to form aldehyde ammonias. Formaldehyde reacts with ammonia in neutral or slightly alkaline solution to form amino ethyl alcohol, methyleneimine, and hexamethylenetetramine.

The action of formaldehyde on ammonia in acid solution is quite different from that occurring in neutral or alkaline solution. In this case formaldehyde appears to act as a methylating agent, forming the three methylated ammonias, mono-, di-, and trimethylamines. These reactions have been studied by Plöchl (4), Cambier and Brochet (5), and especially Werner (6). Werner has shown that all three of the methylamines are formed when ammonia is heated with formaldehyde in acid solution. Dimethylamine is the chief product, trimethylamine being formed in appreciable quantities only when the solution is heated under pressure. Werner assumes the formation of methyleneimine hydrochloride from the ammonium salt and formaldehyde, the methyleneimine reacting with another molecule of formaldehyde by simultaneous oxidation and reduction to yield methylamine hydrochloride.

The methylamine hydrochloride so formed may further react with formaldehyde to form dimethylamine, and in the final stage the dimethylamine reacts further with formaldehyde to give tetramethylenediamine dihydrochloride. Since this is a stable

saturated base, it is not reduced by formaldehyde. The formation of trimethylamine, according to Werner, is due to the decomposition of this base by long heating or heating at temperatures above 100°.

When the amines are distilled from alkaline solution, the tetramethylenediamine is readily hydrolyzed, yielding the volatile base, dimethylamine.

EXPERIMENTAL

Problem—Obviously amines might be formed from amino acids either by decarboxylation or by deamination with subsequent methylation of the ammonia produced. In the first case each amino acid would yield a characteristic primary amine. Such amines might, of course, be methylated to give secondary and tertiary amines, but in each case the amine would have one radical characteristic of the amino acid from which it was formed.

If, however, the first reaction is a deamination of the amino acid, the volatile nitrogen fraction will be composed entirely of ammonia, mono-, di-, and trimethylamines, regardless of the amino acid used.

The present problem, then, is to determine which of these reactions takes place, and to what extent the different amino acids undergo the reaction.

Methods

1. Hydrolysis of Casein in Presence of Formaldehyde—The casein was hydrolyzed in the usual manner by refluxing with 20 per cent hydrochloric acid. The hydrolysis was interrupted at the end of 4 hours, this time being sufficient for the complete hydrolysis of the acid amides with the resulting formation of ammonium chloride (7). The ammonia nitrogen was determined according to the directions of Van Slyke (8). The insoluble humin and excess calcium hydroxide were then filtered off. Trioxymethylene was added to the filtrate and the solution made up to a definite volume with 20 per cent hydrochloric acid. The hydrolysis was then continued at the boiling temperature and aliquots were removed from time to time. These aliquots were steam-distilled to remove the formaldehyde. The ammonia or volatile amines were determined in the same manner as before. The non-volatile amino

nitrogen was determined with the Van Slyke amino nitrogen apparatus.

2. Treatment of Amino Acids in Acid Solution with Formaldehyde—Weighed samples of pure amino acids were mixed with trioxymethylene, made up to definite volume with 20 per cent hydrochloric acid, and refluxed. Aliquots were removed from time to time, the formaldehyde removed by steam distillation, and the volatile nitrogen determined by distillation from alkaline solution into standard acid. Non-volatile amino nitrogen was determined as before.

In order to greatly increase the speed of reaction, similar mixtures of amino acids and formaldehyde were heated to about 160° under pressure in a small bronze autoclave of about 1 liter capacity. Acetic acid was used instead of hydrochloric acid in order not to damage the autoclave. Volatile nitrogen was determined on these preparations after freeing them of formaldehyde.

3. Quantitative Determination of Ammonia, Primary, Secondary, and Tertiary Amines—The method used for the quantitative determination of ammonia, primary, secondary, and tertiary amines was that of Weber and Wilson (9). The total volatile nitrogen is determined by distillation from alkaline solution into a solution of standard acid and titration with standard alkali, methyl red being used as an indicator. The ammonia is then removed by shaking the faintly alkaline solution with powdered yellow mercuric oxide, and filtering. The filtrate is again distilled into standard acid and titrated for total amine nitrogen. The primary amine nitrogen is determined on an aliquot according to the Van Slyke method, the Van Slyke apparatus being modified by using a macro shaking bulb in combination with a micro burette. The remainder of the solution is then treated with nitrous acid, which destroys the primary amines and converts the secondary amines into nitrosamines. After the removal of the nitric oxide, the solution is again distilled into standard acid and titrated, the acid used being equivalent to the unchanged tertiary amines. The secondary amines are then determined either by difference or by reducing the nitrosamine with nascent hydrogen, distilling, and titrating. The method was tried on known mixtures of amines and ammonia, and was found to give reasonably satisfactory results.

4. Preparation of Reinecke's Salt—The double salt of ammonium

thiocyanate and potassium dichromate, $Cr_2NH_3(SCN)_3NH_4SCN-H_2O$, known as Reinecke's salt (10, 11), was found to be more satis-

TABLE I

Separation of Known Mixtures of Ammonia, Methylamine, Dimethylamine, and Trimethylamine by Method of Weber and Wilson

Total N	Ammonia N		Primary amine N		Secondary amine N			Tertiary amine N	
	Found	Present	Found	Present	Found*	Found†	Present	Found	Present
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
28.02	6.82	7.43	6.02	7.28	6.00	8.24	6.77	6.94	6.55
28.02	7.14	7.43	6.56	7.28	6.25	7.51	6.77	6.81	6.55
28.02	7.23	7.43	7.15	7.28	6.35	6.92	6.77	6.72	6.55
28.02	7.31	7.43	6.92	7.28	6.13	7.01	6.77	6.78	6.55

* Determined by reduction of nitrosamine.

† Determined by difference.

TABLE II

Distribution of Volatile Nitrogen in Hydrolysate of 25 Gm. of Casein Hydrolyzed with 20 Per Cent Hydrochloric Acid in Presence of 50 Gm. of Trioxymethylene

Values for $\frac{1}{10}$ aliquots.

Time hydrolyzed with trioxymethylene	Total volatile N	Total amine N		Ammonia N		Primary amine N		Secondary amine N*		Tertiary amine N	
hrs.	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
†	32.65	0.73	2.24	31.92	97.76	0.77	2.36	0.06	0.18	0.05	0.15
1	28.30	27.82	98.30	0.48	1.70	22.29	78.76	6.57	23.22	0.72	2.54
2	27.09	27.25	100.16	−0.16	(0.00)	16.14	59.58	12.47	46.03	0.51	1.88
$4\frac{1}{2}$	38.70	38.33	99.04	0.37	0.96	12.79	33.05	26.95	69.64	0.81	2.09
$10\frac{1}{2}$	46.75	46.23	98.89	0.52	1.11	12.01	25.69	36.66	78.42	1.24	2.65
27	58.66	57.89	98.69	0.77	1.31	6.33	10.79	51.30	87.45	2.59	4.42
70	69.84	69.52	99.54	0.32	0.46	8.56	12.26	57.39	82.17	4.15	5.94

* Determined by reduction of nitrosamine.

† Ammonia fraction removed after 4 hours hydrolysis before addition of trioxymethylene.

factory for the precipitation of secondary and tertiary amines than was chloroplatinic acid.

The salt is prepared by adding finely powdered potassium

dichromate slowly and with stirring to molten ammonium thiocyanate. When an equivalent amount of the dichromate has been added, the mixture suddenly solidifies. The sparingly

TABLE III

Volatile Nitrogen Distribution after Boiling 8 Gm. of dl-Alanine with 25 Gm. of Trioxymethylene Made to 250 Cc. Volume with 20 Per Cent Hydrochloric Acid

Values for $\frac{1}{10}$ aliquots.

Time boiled	Total vola-tile N	Total amine N		Ammonia N		Primary amine N		Secondary amine N*		Tertiary amine N	
hrs.	mg.	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent
$\frac{1}{2}$	17.63	17.47	99.09	0.16	0.91	15.55	89.01	2.12	12.08	0.43	2.46
1	18.40	18.14	98.42	0.26	1.58	13.95	76.90	4.64	25.58	0.20	1.16
2	19.53	19.52	99.95	0.01	0.05	10.77	55.17	9.03	46.26	0.34	1.74
4	26.19	26.43	100.92	−0.24	−0.92	9.11	34.47	16.57	62.69	0.39	1.48
10	34.82	34.83	100.03	−0.01	−0.03	5.84	16.77	28.92	79.56	1.28	3.67
24	40.21	39.92	99.28	0.29	0.78	4.30	10.77	34.99	87.65	2.44	6.11
168	49.86	46.69	99.66	0.17	0.34	7.14	14.37	40.29	77.40	4.09	8.23

* Determined by reduction of nitrosamine.

TABLE IV

Volatile Nitrogen Distribution after Boiling 10 Gm. of Tyrosine with 25 Gm. of Trioxymethylene Made to 250 Cc. Volume with 20 Per Cent Hydrochloric Acid

Values for $\frac{1}{10}$ aliquots.

Time boiled	Total volatile N	Primary amine N		Secondary amine N*		Tertiary amine N	
hrs.	mg.	mg.	per cent	mg.	per cent	mg.	per cent
$\frac{1}{2}$	6.51	6.05	92.93	0.31	4.76	0.15	2.31
1	7.56	6.27	82.94	1.29	17.06	0.00	0.00
2	9.05	5.88	64.97	3.17	35.03	0.00	0.00
4	8.80	5.05	57.39	3.55	40.34	0.20	2.27
9	9.23	3.24	35.10	5.93	64.25	0.06	0.65
24	9.39	1.56	16.61	7.43	79.13	0.40	4.26

* Determined by difference.

soluble Reinecke's salt is freed of any excess of the simple salts by thorough washing with water on a suction filter. The material on the filter, consisting chiefly of the desired salt, is purified by dissolving in the least possible amount of warm ethyl alcohol,

filtering, and pouring the filtrate into about 10 volumes of cold water. After settling, the precipitate of Reinecke's salt is filtered off and dried.

TABLE V

Volatile Nitrogen Distribution after Boiling 10 Gm. of Glutamic Acid Hydrochloride with 25 Gm. of Trioxymethylene Made to 250 Cc. Volume with 20 Per Cent Hydrochloric Acid

Values for $\frac{1}{10}$ aliquots.

Time boiled	Total volatile N	Primary amine N		Secondary amine N*		Tertiary amine N	
hrs.	mg.	mg.	per cent	mg.	per cent	mg.	per cent
$\frac{1}{2}$	7.04	6.19	87.92	0.84	11.94	0.01	0.14
1	7.29	4.94	67.76	2.35	32.24	0.00	0.00
2	10.81	4.05	37.47	6.70	61.97	0.06	0.56
4	16.45	4.65	28.27	11.72	71.24	0.08	0.49
9	22.02	1.24	5.63	20.21	91.78	0.57	2.59
24	26.79	0.73	2.72	24.54	91.61	1.52	5.67

* Determined by difference.

TABLE VI

Volatile Nitrogen Distribution after Boiling 8 Gm. of Cystine with 25 Gm. of Trioxymethylene Made to 250 Cc. Volume with 20 Per Cent Hydrochloric Acid

Values for $\frac{1}{10}$ aliquots.

Time boiled	Total volatile N	Primary amine N		Secondary amine N*		Tertiary amine N	
hrs.	mg.	mg.	per cent	mg.	per cent	mg.	per cent
$\frac{1}{2}$	11.03	8.49	90.90	0.95	8.61	0.02	0.21
1	13.03	9.54	81.33	1.89	16.79	0.10	0.85
2	13.53	7.50	68.87	2.42	22.22	0.00	0.00
4	12.14	6.29	54.18	6.58	56.68	0.13	1.12
10	13.31	4.16	32.76	9.02	71.02	0.45	3.54
24	14.66	1.77	12.77	12.06	87.01	0.81	5.84
168	17.93	1.86	11.24	14.03	84.71	1.59	9.61

* Determined by reduction of nitrosamine.

Experimental Data—The data obtained in this study are given in Tables I to XIV.

DISCUSSION

When the ammonia fraction was removed from a 4 hour casein hydrolysate in the usual manner and the hydrolysis then continued

in the presence of formaldehyde, a second volatile nitrogen fraction was produced which was more than twice as large as the original ammonia fraction (Table II). This confirms the results obtained by Gortner and Holm (2) on the hydrolysis of fibrin and gelatin in the presence of formaldehyde.

TABLE VII

Nitrogen Distribution after Boiling 0.5 Gm. of Ammonium Chloride with 25 Gm. of Trioxymethylene Made to 250 Cc. Volume with 20 Per Cent Hydrochloric Acid

Values for ⅕ aliquots.

Time boiled	Total volatile N	Total amine N		Ammonia N		Primary amine N		Secondary amine N*		Tertiary amine N	
hrs.	mg.	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent
1	26.66	14.57	54.65	12.09	45.35	12.85	48.20	3.30	12.38	0.37	1.39
4	26.58	26.18	98.50	0.40	1.50	14.41	54.21	13.17	49.55	0.46	1.73
22	27.40	27.51	100.40	−0.11	(0.00)	3.29	12.01	25.02	91.31	1.43	5.22

* Determined by reduction of nitrosamine.

TABLE VIII

Nitrogen Distribution after Boiling 0.5 Gm. of Ammonium Chloride with 25 Gm. of Acetaldehyde Made to 250 Cc. Volume with 20 Per Cent Hydrochloric Acid

Values for ⅕ aliquots.

Time boiled	Total volatile N	Total amine N		Ammonia N		Primary amine N		Secondary amine N*		Tertiary amine N	
hrs.	mg.	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent	mg.	per cent
1	27.95	20.31	72.67	7.64	27.33	20.66	73.92	1.02	3.65	0.27	0.97
4	28.40	21.73	76.51	6.67	23.49	21.16	74.51	1.00	3.52	0.26	0.92
22	28.99	20.79	71.71	8.20	28.29	20.16	69.54	0.64	2.21	0.25	0.86

* Determined by reduction of nitrosamine.

When this second volatile nitrogen fraction was analyzed by the method of Weber and Wilson, it was found to consist almost entirely of primary and secondary amines. Traces only of ammonia were found throughout the hydrolysis. Tertiary amines increased slowly to 5.9 per cent of the total volatile nitrogen in 70 hours. If the results are expressed in terms of percentages of the total volatile nitrogen and graphed it will be seen that the curve

for primary amine nitrogen, which predominates at the beginning of the hydrolysis in the presence of formaldehyde, falls rapidly and

TABLE IX

Total Nitrogen Distribution after Boiling 8 Gm. of Alanine with 25 Gm. of Trioxymethylene Made to 250 Cc. Volume with 20 Per Cent Hydrochloric Acid

Values for $\frac{1}{10}$ aliquots.

Time boiled	Volatile N		Non-volatile amine N		Non-volatile non-amine N		
hrs.	*mg.*	*per cent*	*mg.*	*per cent*	*mg.**	*mg.†*	*per cent†*
0‡	0.0	0.0	122.8	100.0	0.0	0.0	0.0
$\frac{1}{2}$	17.5	14.3	73.6	59.9	29.4	31.7	25.8
1	18.1	14.7	52.6	42.8	52.1	52.1	42.4
2	19.5	16.9	30.8	25.1	71.2	72.5	58.9
4	26.4	21.5	14.5	11.8		81.9	66.6
10	34.8	28.3	5.7	4.6	86.2	82.3	66.9
24	39.9	32.5	4.1	3.4	79.7	78.8	64.1
168	49.7	40.5	4.5	3.7	66.3	68.6	55.8

* Determined by Kjeldahl digest.
† Determined by difference.
‡ Theoretical values.

TABLE X

Total Nitrogen Distribution after Boiling 10 Gm. of Tyrosine with 25 Gm. of Trioxymethylene Made to 250 Cc. Volume with 20 Per Cent Hydrochloric Acid

Values for $\frac{1}{10}$ aliquots.

Time boiled	Volatile N		Non-volatile amine N		Non-volatile non-amine N*	
hrs.	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
0†	0.00	0.00	77.35	100.00	0.00	0.00
$\frac{1}{2}$	6.51	8.42	20.08	25.96	50.76	65.62
1	7.56	9.77	11.12	14.38	58.67	75.85
2	9.05	11.70	4.88	6.31	63.42	81.99
4	8.80	11.38	3.26	4.21	65.29	84.41
9	9.23	11.93	0.54	0.70	67.58	87.37
24	9.39	12.14	0.00	0.00	67.96	87.86
168	9.21	11.91				

* Determined by difference.
† Theoretical values.

that the curve for secondary amine nitrogen rises at almost exactly the same rate as the curve for primary amine nitrogen falls. This

TABLE XI

Total Nitrogen Distribution after Boiling 10 Gm. of Glutamic Acid Hydrochloride with 25 Gm. of Trioxymethylene Made to 250 Cc. Volume with 20 Per Cent Hydrochloric Acid

Values for $\frac{1}{10}$ aliquots.

Time boiled	Volatile N		Non-volatile amine N		Non-volatile non-amine N*	
hrs.	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
0†	0.00	0.00	76.32	100.00	0.00	0.00
$\frac{1}{2}$	7.04	9.22	53.20	69.71	16.08	21.07
1	7.29	9.55	39.48	51.73	29.55	38.72
2	10.81	14.16	21.43	28.08	44.08	57.76
4	16.45	21.55	11.40	14.94	48.47	63.51
9	22.02	28.85	4.20	5.50	50.08	65.65
24	26.79	35.10	2.02	2.65	47.51	62.25
168	26.80	35.12	2.32	3.04	47.20	61.84

* Determined by difference.

† Theoretical values.

TABLE XII

Total Nitrogen Distribution after Boiling 8 Gm. of Cystine with 25 Gm. of Trioxymethylene Made to 250 Cc. Volume with 20 Per Cent Hydrochloric Acid

Values for $\frac{1}{10}$ aliquots.

Time boiled	Volatile N		Non-volatile amine N		Non-volatile non-amine N*	
hrs.	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
0†	0.0	0.0	94.1	100.0	0.0	0.0
$\frac{1}{2}$	9.3	9.9	21.3	22.6	63.5	67.5
1	11.7	12.4	20.4	21.7	62.0	65.9
2	10.9	11.6	11.9	12.6	71.3	75.8
4	11.6	12.3	4.2	4.5	78.3	83.2
10	12.7	13.5	2.2	2.3	79.2	84.2
24	13.9	14.8	1.7	1.8	78.5	83.4
168	16.5	17.5	3.7	3.9	73.9	78.5

* Determined by difference.

† Theoretical values.

would indicate that the secondary amines were being formed from the primary, hence were not primary reaction products of the protein and formaldehyde.

In order to compare the behavior of different amino acids with

each other and with casein, the four amino acids, alanine, glutamic acid, tyrosine, and cystine, were boiled with 10 per cent formalde-

TABLE XIII

Nitrogen Distribution after Autoclaving Ammonium Chloride and Four Amino Acids with 25 Gm. of Trioxymethylene Made to 250 Cc. Volume with 5 Per Cent Acetic Acid at ±180° for 6 Hours

The values are expressed as per cent of total nitrogen.

Material	Primary amine N	Secondary amine N	Tertiary amine N	Total volatile N	Total non-volatile N
	per cent	per cent	per cent	per cent	per cent
Ammonium chloride..............	0.00	2.91	97.09	100.00	0.00
Alanine.........................	0.69	2.07	24.88	27.64	72.36
Tyrosine........................	0.00	1.77	45.43	47.20	52.80
Glutamic acid...................	0.87		41.12	41.99	58.01
Cystine.........................	1.25	2.35	65.61	69.21	30.79

TABLE XIV

Comparison of Trimethylamine with Tertiary Amines Prepared from Four Amino Acids and from Ammonium Chloride

Amine	Compound with Reinecke's salt				Chloroplatinate from amine from Reinecke's salt compound	
	Total N	Amine N	Melting point	Molecular weight*	Melting point	Platinum content
	per cent	per cent	°C.		°C.	per cent
Trimethylamine†............	9.77	3.26		430		37.68
" ‡............	10.35	3.19	283–284	439	220	
From NH₄Cl................	10.58	3.27	281–282	428	220	36.12
" alanine..............	10.79	3.28	281–282	427	220	38.63
" tyrosine..............	10.65	3.29	280–282	425	220	36.99
" glutamic acid........	10.81	3.34	281–282	419		
" cystine..............	10.74	3.36	281–282	417		

* Determined from amine nitrogen content.
† Theoretical values.
‡ Experimental values.

hyde (trioxymethylene) in 20 per cent hydrochloric acid solution. Aliquots were removed from time to time and the volatile nitrogenous compounds were distilled from alkaline solution as in the

case of the casein, except that due to the absence of acid amides no preliminary removal of an ammonia fraction was necessary. These four amino acids represent, respectively, four distinct types of naturally occurring amino acids; (1) monoaminomonocarboxyl, (2) monoaminodicarboxyl, (3) an amino acid with an aromatic group, and (4) one of the hexone bases.

From Tables II to VI it is evident that the effect of boiling with formaldehyde in the presence of acid upon the distribution of the volatile nitrogen is essentially the same for all the amino acids used and for casein. Since this nitrogen distribution appeared to be very similar to that which might be expected from the methylation of ammonia by formaldehyde according to Werner's explanation, it seemed advisable to determine the nitrogen distribution in a similar manner using an ammonium salt rather than an amino acid. For this purpose 0.5 gm. of ammonium chloride was boiled with 25 gm. of trioxymethylene in 250 cc. of 20 per cent hydrochloric acid and the nitrogen distribution determined after various lengths of time (Table VII). It is evident that after the first 2 hours the distribution was practically identical with those of the amino acids and of casein. Obviously the differences in the first 2 hours are due to the fact that in the case of the amino acids no volatile nitrogen is present at the beginning of the reaction, while in the case of the ammonium chloride the nitrogen is all potentially volatile throughout the reaction. The evidence seems to indicate that amino acids in acid solution are to a certain extent deaminized by formaldehyde and that the ammonia so formed is progressively methylated to methyl-, dimethyl-, and finally, if the reaction is carried on long enough, to trimethylamine. The fact that little or no ammonia was detected in any of the preparations may be explained by assuming that the methylation reaction is more rapid than the deamination, in which case the ammonia would be methylated as soon as it is formed.

The possibility of using acetaldehyde in the place of formaldehyde was investigated in the case of the ammonia reaction. It was found to be unsuitable, since in all cases the solutions rapidly turned black with the precipitation of a black horn-like material, probably a condensation or polymerization product, after which no further change in the nitrogen distribution took place. From Table VIII, however, it appears that acetaldehyde, during the

short time before its reactivity is destroyed, alkylates ammonia even more rapidly than does formaldehyde. This reaction was not further investigated, but it appears that it might be useful in the preparation of primary ethylamine.

The total nitrogen distribution after boiling the amino acids with formaldehyde was also studied, the total nitrogen being divided into three fractions: (1) volatile nitrogen, (2) non-volatile amino nitrogen, (3) non-volatile non-amino nitrogen. The volatile fraction includes, of course, the volatile amines and ammonia whose determination have been discussed. The non-volatile amino nitrogen corresponds, no doubt, to the unchanged amino acid, and is determined with the Van Slyke amino nitrogen apparatus on the residue left after distilling off the volatile nitrogen fraction. The non-volatile non-amino fraction is determined by difference and is probably due to the presence of methylene derivatives of the amino acids, or to methylated amino acids. Tables IX to XII show the total nitrogen distribution on the four amino acids under investigation.

Except in the case of tyrosine, the volatile nitrogen continued to increase gradually as long as the solutions were boiled. The non-volatile amino nitrogen in each case, however, reached a constant minimum value after a comparatively few hours, showing that the volatile nitrogen, after the first few hours at least, is derived from the non-volatile non-amino fraction. This would indicate that if ammonia is the primary volatile product formed, it is not formed by a simple hydrolytic deamination of the amino acid, but through some intermediate compound.

On the assumption that the volatile amines are formed by the liberation of ammonia and the subsequent methylation of the ammonia, the end-product should be trimethylamine regardless of the amino acid used. In order to prove this contention similar solutions of amino acids and formaldehyde in acetic acid instead of hydrochloric acid were heated under pressure at about 180° for 6 hours. The nitrogen distributions on these preparations are shown in Table XIII. In each case practically all of the volatile nitrogen is in the tertiary form.

The tertiary amine was precipitated as the Reinecke's salt compound and compared with the same compound from pure trimethylamine. The total nitrogen, amine nitrogen, melting points,

and molecular weights calculated on the basis of the percentage of amine nitrogen were all in close agreement with the theoretical and experimental values for trimethylamine (Table XIV). The chloroplatinates prepared from the amines liberated from the Reinecke compounds also showed the correct melting points and platinum contents for trimethylamine.

SUMMARY

Casein, ammonium chloride, and the four amino acids, alanine, cystine, glutamic acid, and tyrosine, were boiled with formaldehyde in 20 per cent hydrochloric acid solution and the volatile and total nitrogen distributions determined, after different lengths of time. The same four amino acids and ammonium chloride were autoclaved for 6 hours at about 180° with formaldehyde in 5 per cent acetic acid solution, and the same nitrogen distributions determined. The Reinecke's salt compounds and the chloroplatinates of the tertiary amines formed in the autoclave experiments were prepared and compared with the corresponding compounds of trimethylamine.

The data support the following conclusions:

1. Volatile amines to the extent of from 12 to 40 per cent of the total nitrogen are formed when amino acids are boiled with formaldehyde in acid solution.

2. The distribution of primary, secondary, and tertiary amines in these preparations is essentially the same as that when ammonium chloride is boiled with formaldehyde under the same conditions.

3. The rate of deamination of amino acids in acid solution is greatly increased by the presence of formaldehyde.

4. The volatile nitrogen is probably not produced by simple deamination of the amino acid, but through some intermediate non-volatile compound containing no primary amino group.

5. The end-product of the volatile nitrogen fraction for the amino acids studied is trimethylamine.

6. No evidence was obtained for the formation of amines by decarboxylation of amino acids in the presence of formaldehyde.

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STUDIES ON THE DIELECTRIC CONSTANT OF PROTEIN SOLUTIONS

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CONTENTS

The recent development of the theory of dielectric constants in relation to molecular properties has led to a considerable increase of knowledge in regard to the nature of many organic and inorganic compounds. A succinct account of the theory itself has been given in his recent book "Polar Molecules" by Debye (1), to whom the subject is so largely due. A discussion of the applications of the theory to problems of molecular structure and behavior in the light of experimental work on dielectric constants is given in two recent reviews by Smyth (2) and Williams (3). These applications have hitherto been to fairly simple compounds of moderate molecular weight. Certain implications of the theory, however, make it of considerable interest to investigate in this connection a class of very exceptional substances, the proteins. The extremely large size of the protein molecules, the great number of polar groups which they contain, and the possibility of their existence as *Zwitter Ionen* suggest unusual properties in relation to the dielectric constant. For this reason the present study was undertaken.

I

Debye's theory is based on the classical relations of the electro-static field. According to Maxwell's procedure, the dielectric constant ϵ of the medium is defined in terms of the vectors D and E, expressing respectively the electric displacement and electric intensity of the field, by the equation:

(1) $D = \epsilon E^*$

* For convenience a list of symbols is appended at the end of the paper.

In the case of crystalline substances the value of ϵ varies according to the orientation of the medium with respect to the field and is in general expressible by the components of a tensor.   In the case of isotropic substances on the other hand ϵ is independent of direction and is to be regarded as a scalar quantity.   It is with substances of the latter class that the theory is concerned.   The existence of a dielectric constant different from unity (the value for free space) is explained classically by a polarization of the medium so that every element of volume dV assumes an electric moment IdV in the electric field; I being given by the expression

(2) $D = E + 4\pi I$

Now I, the electric moment per unit volume, is itself made up by contributions of the individual molecules and is equal to nm where n is the number of molecules per unit volume and m is their mean electric moment in the direction of the field.   Based on these classical relations, the development of the theory now consists of two parts.   The first of these is the derivation of expressions for the mean moment of the molecules in terms of molecular constants and the vectors D and E defining the field; the second is the elimination of D and E between these expressions and equations (1) and (2) so as to obtain a final result for ϵ in terms of molecular constants.

In regard to the first part of the problem it is assumed that there are two classes of molecules.   In Class 1 the molecules have no permanent electric moment, but when subjected to an electric force F they are supposed to acquire an induced moment $m_0 = \alpha_0 F$ as the result of a displacement of charge within the molecule. In Class 2 the molecules possess a permanent electric moment

μ due to their structure in addition to any induced moment they may acquire in a field. If molecules of Class 2 are not subjected to any force they will be distributed at random in space and their mean moment in any direction will be zero, but if they are brought into an electric field they will tend to orient to an extent determined by the strength of the field and the temperature (as affecting the random heat motion). By applying the Maxwell Boltzman distribution law it can be shown that if the molecules are acted on by a constant force F the mean moment in the direction of F due to orientation is given by

$$(3) \qquad \overline{m} = \mu \left(\text{cotgh.*} \frac{\mu F}{KT} - \frac{KT}{\mu F} \right)$$

   * Cotgh. is an abbreviation for hyperbolic cotangent.

where T is the absolute temperature and K is a constant $= 1.37 \times 10^{-16}$. To this must be added, of course, the induced moment $m_0 = \alpha_0 F$ in order to obtain the total moment. In the development of the theory a simplification is introduced by replacing the right hand side of equation (3) by its first approximation which gives finally for m

$$(4) \qquad m = \left(\alpha_0 + \frac{\mu^2}{3 KT} \right) F$$

The adequacy of this approximation is justified by the small values of F with which we are concerned in practice (see Debye (1) p. 78) and by the experimental fact that measurements of the dielectric constant are independent of the strength of the field, showing that the mean moment of the molecules is actually proportional to F. Only in the case of the strong fields surrounding ions can we expect the approximation to fail (the so called saturation effect).

The results in regard to molecules of Class 2 are somewhat different if we consider the important case where they are subjected to an alternating force $F = F_0 e^{i\omega t}$.[1] In this case if the

[1] According to the usual practice in the treatment of alternating fields we employ the operator e^{i0}. Only the real part of the quantity resulting from the use of the operator is to be considered; e.g., $F_0 e^{i\omega t}$ stands for $F_0 \cos.\omega t$.

frequency $\frac{\omega}{2\pi}$ is high enough to be comparable with the period of
rotation of the molecules we no longer have statistical equilibrium
and in place of the Maxwell Boltzman law it is necessary to make
use of another distribution law, based on the Einstein theory of
Brownian movement and involving the so called relaxation time
of the molecules.   This quantity, denoted by τ, is the time re-
quired for the orientation of the molecules to fall to $\frac{1}{e}$ of its equi-
librium value in a constant field if the field is suddenly removed.
It can be shown that $\tau = \frac{\zeta}{2\,KT}$ where ζ is a constant measuring
the frictional resistance offered to the rotation of the molecules.
According to Stokes' calculation for the case of a spherical particle
of radius a in a medium of viscosity η, $\zeta = 8\pi\eta a^3$.   If we assume
the molecules to behave as spheres we therefore obtain the result:

$$(5) \qquad\qquad \tau = \frac{4\,\pi\,\eta\,a^3}{KT}$$

By making use of this treatment it is found that the mean moment
due to orientation of the Class 2 molecules subjected to the
alternating force F is given by

$$(6) \qquad\qquad \overline{m} = \frac{\mu^2\,F}{3\,KT\,(1 + i\,\omega\,\tau)}$$

to the first order of approximation as in the case of the equilibrium
condition.   To this moment due to orientation we must add, of
course, the induced moment as in the case of the constant field.
The fact that $\overline{m}$ is complex shows that there is a phase difference
given by the angle $\phi = \tan.^{-1}\omega\tau$ between $\overline{m}$ and F.   This, of
course, simply means that the molecules lag behind the rapidly
changing force so that the maximum values of $\overline{m}$ and F do not
occur at the same instant.   As we shall see this lag is connected
with energy absorption by the dielectric.   These considerations
show that in all cases considered the mean moment whether in-
duced or due to orientation or both, is to the first order of approxi-

mation proportional to the effective electric intensity F acting on the molecules, so that we may write quite generally:

$$(7) \qquad\qquad m = \alpha F$$

where α is a constant which can be expressed in any particular case in terms of molecular constants according to the relations given above.

The remaining part of the problem now consists in combining these results with the equations of the field to obtain a final expression for α in terms of ϵ. This expression will embody such information as can be obtained from a knowledge of the dielectric constant in regard to the molecular properties of the medium. At this point, however, there is a difficulty owing to the fact that the effective internal force F acting on the molecules cannot be identified with D or E of the field equations and cannot in general be determined in terms of them so as to allow the required eliminations. It is only possible to calculate F in terms of D and E if we neglect the contribution due to the molecules immediately surrounding the point at which F is to be determined. In the case of gases and probably also non-polar liquids or solutions in non-polar solvents and in the special case of a cubic crystal this contribution may actually be shown to vanish, although this will not be so generally. But whenever this approximation in regard to F can be made we obtain the expression

$$F = E + \frac{4\pi}{3} I$$

From this, together with the previous results, is obtained finally the Clausius Mosotti relation between ϵ and the so called molar polarization P:

$$(8) \qquad\qquad \frac{\epsilon - 1}{\epsilon + 2} \frac{M}{\rho} = \frac{4\pi N \alpha}{3} \equiv P$$

where ρ is the density of the medium, M is the molecular weight, and N is Avogadro's number ($= 6.06 \times 10^{23}$). In case we are dealing with a solution in which the mol fractions, molecular weights, and molar polarizations of n components are $f_1, f_2, \ldots f_n$,

M_1, M_2, ... M_n, and P_1, P_2, ... P_n, respectively, this expression becomes

$$(9) \qquad \frac{\epsilon - 1}{\epsilon + 2} \frac{f_1 M_1 + f_2 M_2 \ldots + f_n M_n}{\rho} = f_1 P_1 + f_2 P_2 \ldots + f_n P_n$$

If the mol fractions f are replaced by concentrations c, expressed in gm. per cc., equation (9) takes the form

$$(9') \qquad \frac{\epsilon - 1}{\epsilon + 2} = c_1 \frac{P_1}{M_1} + c_2 \frac{P_2}{M_2} \ldots + c_n \frac{P_n}{M_n}$$

The most obvious application of the theory is to determine whether or not a molecule is polar, and if so, the magnitude of its electric moment. For this purpose the dielectric constant of the substance in the vapor state or in solution in a non-polar solvent is measured by any suitable method and the molar polarization calculated by the Mosotti relation. Now in the case of a polar molecule this will be (by equations (4) and (8)):

$$(10) \qquad P = \frac{4 \pi N}{3} \left(\alpha_0 + \frac{\mu^2}{3 KT} \right)$$

and will vary with the temperature. By determining P at two or more temperatures it is therefore possible to eliminate α_0 and obtain the value of μ. It ought to be pointed out that α_0 represents the optical polarization corresponding to fields of such high frequency that the molecules do not have time to orient at all and may be determined independently from optical data. Much recent work of this kind bearing on molecular structure is described by Smyth and Williams (2, 3).

Another application of the theory is in regard to the relaxation time τ of the molecules. In this connection it is plainly necessary to study the dielectric constant in an alternating field over a range of frequency embracing the characteristic molecular frequency $\frac{1}{\tau}$, for only in this range does the mean moment and consequently the dielectric constant involve τ (see above). A distinguishing feature of this region lies in the behavior of the dielectric constant, an expression for which may be obtained by applying the Mosotti

relation and expressing the molar polarization in terms of the molecular constants (see equations (8) and (6)).

$$(11) \qquad \frac{\epsilon - 1}{\epsilon + 2} \frac{M}{\rho} = \frac{4 \pi N}{3} \left(\alpha_0 + \frac{\mu^2}{3 KT (1 + i \omega \tau)} \right)$$

Consideration of this result shows that as $\omega \tau$ approaches zero the imaginary part of the right hand side drops out and the equation reduces to the ordinary expression for polar molecules in a static field; on the other hand, as $\omega \tau$ becomes infinite the right hand side also becomes real but the equation now reduces to the expression for the optical case where the frequency is too great for the molecules to orient. In the intermediate range where ω is comparable with τ lies the effect in which we are interested. Here the right hand member is complex and ϵ itself is therefore made up of a real and an imaginary part which we call ϵ' and ϵ'' respectively.

From an analysis of the case of a condenser filled with the dielectric and subjected to a potential difference $V_0 e^{i\omega t}$ of frequency within the range in question it can be shown that ϵ' is equal to the ratio of the capacity of the condenser filled with the medium to that of the empty condenser. This, however, is simply the apparent value of the dielectric constant as determined experimentally by any of the actual methods such as Nernst's bridge method or any of the various types of resonance method. Within the range ϵ' has everywhere a value intermediate between the static value ϵ_1, which it assumes for small values of $\omega \tau$, and the optical value ϵ_0, which it assumes for large values of $\omega \tau$. The region in question is, therefore, characterized by anomalous dispersion.

Associated with this is an absorption phenomenon related to ϵ''. Further consideration of the condenser shows that ϵ'' can be identified with an *apparent* specific conductivity of the dielectric given by

$$(12) \qquad \Lambda = \frac{\epsilon'' \omega}{4 \pi} \text{ E.S.U.}$$

This amount of conductivity in the dielectric would give rise to exactly the same dielectric losses as are actually produced by absorption and would be in no way distinguishable from it under

the conditions of the measurement.   The physical interpretation of the absorption is to be found as already mentioned in the phase difference between the internal force F and the mean moment $\bar{m}$.   For very large and very small values of $\omega\tau$, ϵ'' becomes vanishingly small, and the range in which absorption is appreciable is closely coincident with that of the anomalous dispersion.

In cases which satisfy the requirements of the Mosotti relation we can, therefore, apply the theory to calculate values of the relaxation time τ of the molecules from measurements of the dielectric constant in the region of anomalous dispersion, or conversely from values of τ computed from its definition and Stokes' formula we can predict the position of the anomalous dispersion, assuming the formula to hold.   For this purpose the following equations given by Debye ((1) p. 94) are fundamental.

$$(13) \qquad\qquad \epsilon' = \epsilon_0 + \frac{\epsilon_1 - \epsilon_0}{1 + x^2}; \quad \epsilon'' = \frac{(\epsilon_1 - \epsilon_0)\, x}{1 + x^2}$$

where

$$x = \frac{\epsilon_1 + 2}{\epsilon_0 + 2}\, \omega\, \tau$$

On the other hand to cases which do not fulfil the Mosotti conditions, *e.g.* if we are concerned with measurements on polar liquids, the results given above cannot be expected to apply quantitatively. Nevertheless, the general character of the situation ought not to be very different.   In fact the work of a number of observers on polar liquids shows the presence of anomalous dispersion at about those frequencies at which we might expect it if we applied the Mosotti relation directly, in these cases at wave-lengths ranging from a few meters to a few cm.   At such high frequencies it is difficult to obtain absolute measurements of capacities and conductivities and most of the work is incomplete from the point of view of the theory.

The most complete results are those of Mizushima on glycerol and several alcohols, discussed at some length by Debye ((1) pp. 95–108).   By a comparative method Mizushima was able to obtain values for both ϵ' and Λ, and hence also for ϵ'' by equation

(12), for these substances at a variety of temperatures at the three wave-lengths 50, 9.5, and 3.08 meters and to establish the existence of a region of anomalous dispersion. Although these liquids are all polar, the agreement between theory and observation is surprizingly close. The predicted effect of temperature in decreasing the relaxation time (according to equation (6)) is verified. Indeed, it is found that calculated values of ϵ' and ϵ'' agree with the observed values to within only a few per cent, which suggests that the error involved in applying the Mosotti relation to polar liquids may not be very great.

<center>II</center>

Let us turn now to the question of the proteins in relation to dielectric constants. We will consider the case of solutions, since this is the condition in which most proteins can be studied best. Inasmuch as dielectric constants may be expressed according to Debye's theory through molar polarizations in terms of the molecular constants α_0, μ, and τ, we will direct our attention to these. Consider first α_0, the optical polarization. It has been calculated that if we could regard a molecule as a conducting sphere α_0 would simply be equal to the cube of the radius. Although any such picture is obviously inadequate it does in fact appear that in a large number of cases α_0 is actually roughly proportioned to the molecular volume as determined from the constants of the van der Waals equation applied to the substance in the gaseous state. The reason for this has been discussed at some length by Debye ((1) p. 17) on the basis of a simple physical model. On general grounds it is perhaps only what we might expect since the number of displaceable charges in a molecule ought to be roughly proportional to molecular weight. But whatever the interpretation this fact would certainly imply very large values of the optical polarization for a protein as compared with substances of ordinary molecular weight.

The permanent moment μ of the protein molecules is connected with the much mooted question of their structure. Built up as they are of large numbers of amino acids the protein molecules must contain a great many polar groups such as pyrrole, benzene, and imidazol rings in addition to various other substituents in-

cluding carboxyl, amino, and amide groups.[2]   All these will, of
course, have permanent moments of their own which will add up
vectorially, but whether their arrangement in the molecule will
be such that the groups largely neutralize one another as in the
case of a random distribution, or supplement one another to
produce a large net moment will depend on the particular protein
considered and cannot be predicted generally.

Apart from the question of polar groups, since all the proteins
known are ampholytes it is natural to suppose that they may ionize
to form internal salts according to Bjerrum's concept of the *Zwitter
Ion*.   In general this would be expected to give rise to very large
moments, depending on the number and proximity of the free acid
and basic groups.   This may be illustrated by a numerical case.
Consider a hypothetical protein of gram-molecular weight 100,000.
Most proteins in solution have a partial specific volume of about
0.7 cc., that is to say, one additional gm. of protein added to a
large amount of solution will increase the volume by 0.7 cc.   On
the basis of this figure the individual molecules of the above
protein would occupy 12×10^{-20} cc.   This corresponds to a
molecular radius (assuming the molecules to be spheres) of about
3×10^{-7} cm.   Two elementary charges (4.77×10^{-10}), one posi-
tive and one negative, separated by, say, half this distance have a
moment of 72×10^{-18} electrostatic units.   This is about 44 times
as great as the observed moment of ethyl alcohol dissolved in
carbon tetrachloride and about 12 times that of *o*-dinitrobenzene.
Yet this value would correspond to only a single acid and a single
basic group in the molecule.   As a matter of fact the titration
curves of proteins show them to be of a much higher valence than
this.   Even in the case of zein, one of the most inert of all proteins
studied, the number of free carboxyl groups appears to be thirty
and that of free basic groups about sixteen (see Cohn (5)).   It
must not be forgotten, however, that the effect of higher valence,
leading to a greater number of positive and negative charges in the
Zwitter Ion, might be offset by the arrangement of the acid and
basic groups in the molecule.

Consider finally, the question of the relaxation time τ of the

[2] A discussion of the composition of the proteins is given by Cohn (4);
see also (5).

dissolved protein molecules. This will vary, of course, with temperature and with the viscosity of the solution. If we assume that the molecules are spheres we can apply equation (6) above, which shows that for a given temperature and viscosity the relaxation time is proportional to the volume of the molecules. For molecules of such enormous size as the proteins this would mean very large values as compared with other substances. If we consider again a protein of molecular weight 100,000 and in a solution at 50° and of viscosity 0.01 C.G.S. unit (that of water at room temperature) we obtain for τ the value 8×10^{-8} sec. This is equivalent to a wave-length of about 25 meters in free space, and is over 3000 times as great as the value for water molecules given by Debye.

So far as the writer is aware, the only previous experimental work on this subject is that of Fürth (6), who investigated the dielectric constant of various substances of biological interest, including several of the sugars, urea, the amino acid glycocoll, and two proteins, commercial gelatin and albumin obtained from Kahlbaum. These substances were studied at room temperature in aqueous solution at various concentrations by means of the so called second method of Drude. This method consists in determining the length of standing electric waves on parallel wires immersed in the liquids. The measurements were made at a fixed frequency corresponding to a wave-length of 76 cm. in the air. The results showed the solutions of the sugars and the proteins to have dielectric constants lower than pure water by an amount increasing with the concentration. On the other hand, it was found that solutions of urea and glycocoll had higher dielectric constants than water, reaching values as great as 90. This would indicate that these molecules are highly polar. The explanation of the low dielectric constants observed in the case of sugar and the proteins is not clear on the basis of these measurements alone. It is certainly surprizing that the two proteins should behave so differently from the amino acid, to which they are so closely akin.[3] In accordance with Debye's theory, we might explain this behavior on the basis of a saturation effect (mentioned above), a low value

[3] Other observers have obtained equally high values for the dielectric constants of amino acids. See Blüh (7), Walden and Werner (8), and Hedestrand (9).

of the polarization of the solute, or anomalous dispersion; but in order to settle this question it would be necessary to have further data at other temperatures and lower frequencies. The present study is the result of an attempt to obtain such data.

III

A serious difficulty arises in work at lower frequencies on account of the conductivity of most proteins in solution. It is well known, of course, that increasing conductivity of the dielectric results in a progressive dulling of the settings involved in all capacity determinations. The effect is the more apparent the lower the frequency at which the measurements are made, its magnitude being inversely proportional to the frequency, approximately. This difficulty due to conductivity is involved in all determinations of dielectric constants whether made by a capacity bridge method or by resonance. Even in the case of distilled water it introduces an appreciable error and uncertainty at a frequency as high as about 10^6, corresponding to a wave-length of 300 meters. In the present case, where owing to the question of dispersion it is important to extend the measurements to frequencies as low as we can, it is desirable to select for study a protein having the least possible amount of conductivity, the more so also in view of the absorption with which we may expect to be involved.

For this reason it was decided to study the plant protein zein, prepared from the seeds of maize. Zein belongs to the class of prolamines which are readily soluble in mixtures of alcohol and water where the conductivity is low. It is highly soluble in 95 per cent methyl alcohol, 85 per cent ethyl alcohol, or 70 per cent n-propyl alcohol, forming clear, yellowish, viscous solutions (10). It has a minimal molecular weight of about 100,000 as determined from the results of analyses of the amino acid content (11). For the present purpose it was dissolved in 70 per cent n-propyl alcohol where it appeared to be very stable. Solutions heated to 80° showed no sign of precipitation or change of measured properties when returned to room temperature. The material was kindly supplied by Dr. Dill, from a preparation obtained from the Connecticut Agricultural Experimental Station, to whom the author is indebted. Measurements of density, viscosity, and specific conductivity made on the solvent and on a solution containing 0.131

gm. of zein per cc. at 25° (determined by dry weight) gave the results presented in Table I. The figures given for density and viscosity may be compared with the values for water at 25° (0.9973 and 0.0089 c.g.s. units respectively). The conductivity of the solvent was governed by that of the distilled water used. This varied a good deal but was in the neighborhood of 3–6 × 10⁶ E.S.U., a figure of course much larger than that for the best samples of specially prepared conductivity water. In view of the insolubility of the zein in water, its method of preparation, its low conductivity, and the fact that an analysis showed it to be very nearly ash-free, it was probably not far from its isoelectric point, corresponding to a hydrogen ion activity of about 10^{-6}.

TABLE I

Data in Regard to Density, Viscosity, and Conductivity of 70 Per Cent n-Propyl Alcohol and of a 13.1 Per Cent Solution of Zein in This Solvent

	70 per cent n-propyl alcohol*		Zein solution (0.131 gm. per cc. at 25°)†	
	25°	50°	25°	50°
Density..........................	0.8789	0.8594	0.9210	0.9025
Viscosity (c.g.s. units)............	0.0295	0.0153	0.543	0.180
Conductivity (E.S.U. × 10⁻⁶)......	3.45		20.0	

* Mol fractions are as follows: alcohol, 3.5; water, 6.5.

† Mol fractions are as follows: alcohol, 3.5; water, 6.5; zein, 7.05×10^{-5}.

Two methods of determining dielectric constants were employed. The first of these, best suited for use at frequencies greater than about 10^7 (corresponding to a wave-length of 30 meters in free space) has been recently described by the writer (12). It is a resonance method, based on the principle that the natural period of a small all metal circuit is proportional to the square root of the dielectric constant of the surrounding medium. If by its reaction on an oscillator we determine the resonant frequency of such a circuit or "resonator" first in air and then immersed in a dielectric medium we obtain the dielectric constant of the latter directly by taking the square of the ratio of the corresponding frequencies. With the help of a crystal oscillator as a standard it is possible to determine these frequencies and consequently the dielectric con-

stant very accurately, provided the conductivity (or absorption, which is equivalent to it) of the dielectric is small.

As the conductivity becomes larger the accuracy of the determinations, of course, falls off, and certain departures from the simple theoretical relationship between dielectric constant and frequency are to be expected. These may, however, be shown experimentally to be unimportant, at a wave-length of about 5 meters, up to conductivities at least 25 times that of distilled water. In practice, so long as the settings remain reasonably sharp, the error due to this cause is found to be well within the experimental error in making settings (see Wyman (12)). This condition was certainly satisfied by the measurements on zein, though it was apparent that there was an appreciable amount of absorption or conductivity.

Circuits resonant at a wave-length of 4 or 5 meters in a medium of dielectric constant about 30, a round value in the case of the zein solutions, are conveniently small, and may be satisfactorily immersed in about 30 cc. of liquid. The resonator actually used in the present case consisted of two concentric brass cylinders joined by a rigid brass loop. This was suspended by a fine thread in the protein solution contained in a test-tube, and the tube itself was sunk in a water bath of desired temperature. This method, though of great accuracy and convenience and possessing the advantage of giving absolute results, is inconvenient for work at lower frequencies owing to the size of the resonators involved. A resonator similar to that used at 4 meters but having a period of 10^6 ($\lambda = 300$ meters) would require about 2 liters of solution for immersion. To obtain such a quantity of material in the case of a substance so troublesome to prepare as zein would offer a serious difficulty. For this reason a second method was devised, requiring much less material.

The second method like the first is based on resonance. This time, however, the frequency is fixed and the unknown dielectric constant is determined from the settings of a variable condenser. The principle underlying this method can be most readily understood in connection with the circuit diagram shown in Fig. 1. The solution to be measured is placed in a small glass cell of about 25 cc. capacity (represented in Fig. 2). This is introduced into Circuit 1, which we call the resonator, in parallel with a variable

condenser.   The resonator itself is inductively coupled with a vacuum tube oscillator (Circuit 2 of the diagram), which is in turn coupled with a piezoelectric oscillator (Circuit 3) operating
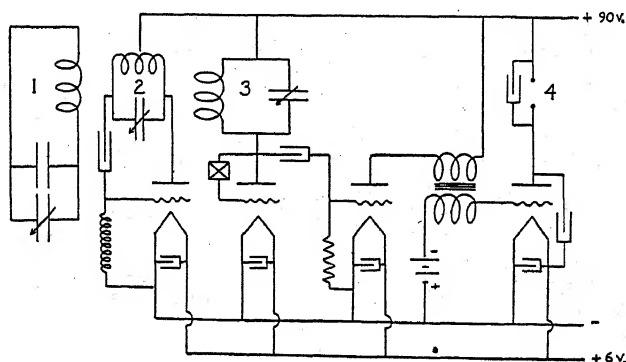
FIG. 1. Circuit diagram to illustrate the second method used to measure dielectric constants.   1 = resonator, 2 = oscillator, 3 = piezoelectric oscillator, 4 = telephone.
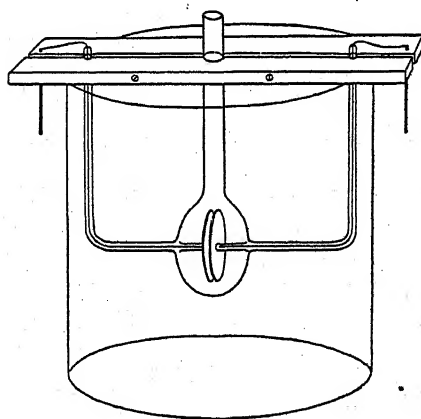
FIG. 2. Capacity cell used in the second method of determining dielectric constants.

with great constancy at a wave-length of 65 meters.   The rest of the arrangement shown in Fig. 1 simply serves to amplify and make audible beats between the two oscillators.   To make a

measurement Circuit 2 is set oscillating and adjusted until beats between fundamentals or harmonics of the two oscillators are heard at some suitable frequency, which is measured with a precision wave meter and can be determined with very great accuracy in terms of the fundamental of the piezoelectric oscillator. Then by means of the variable condenser the resonator is tuned to this frequency, the indication of resonance being the reaction on the oscillator (Circuit 2) as measured by a sudden change in its plate current. From the corresponding setting of the variable condenser the unknown dielectric constant is obtained directly as a result of a previous calibration with known liquids. One great advantage of the method lies in the fact that it is possible to listen to the beat note throughout the course of the measurement and be sure that there is no drag on the oscillator while the resonator is being tuned. It also definitely fixes the frequency of the measurements. Since beats may be heard over a wide range of frequency on both sides of the fundamental of the piezoelectric oscillator this feature of the method introduces no real restriction on frequency. Unfortunately, it is not possible to measure absorption by this method, although from the sharpness of the reaction it is possible to get a qualitative indication of its magnitude. During the measurements the small cell containing the solution is immersed in a water bath of desired temperature (controlled by ice or a heating coil) as indicated in Fig. 2. It ought to be mentioned perhaps that in order to provide for the necessary range of frequency both resonator and oscillator (Circuit 2) were supplied with interchangeable inductances.

At each of the frequencies selected for the measurements (λ = 260, 195, 152, 97.5, and 48.8 meters) it was necessary to carry out a calibration of the resonator. The liquids employed for this purpose were mixtures of ethyl alcohol and water for each of which the dielectric constant was determined at the time to about 0.1 per cent at a wave-length of about 4 meters by the earlier method which gives absolute values. Successive calibrations of the apparatus showed no change. Since the solutions showed an appreciable amount of absorption, some of which was undoubtedly due to actual conductivity, it was necessary to consider the effect of this on the values obtained for the dielectric constant, or more correctly its real part, which is what we actually measure where

we are concerned with absorption (see Section I); for it is known that conductivity and absorption in the dielectric may lead to systematic errors in the determination of this quantity. The magnitude and sign of these errors will depend on the properties of the circuit, and owing to the uncertainties of any circuit analysis at the frequencies involved they cannot be calculated satisfactorily from theory. In order to examine this matter small amounts of ammonium sulfate were introduced into the calibrating liquids in order to increase the conductivity and produce the equivalent effect of absorption. It was assumed, as was done by Mizushima in a similar calibration in his method, that this did not change the dielectric constant by an amount significant for the accuracy of the measurements, about 1 per cent. When the liquids were in-

TABLE II

Results Showing Absence of Any Effect of Conductivity on Values of Dielectric Constants Measured by the Second Method

Wave-length, *meters*	260	195	152	97.5	48.8
Dielectric constant of ethyl alcohol-water mixture	41.2	40.9	40.8	41.0	40.8
Dielectric constant of same + $(NH_4)_2 SO_4$*	40.2	40.9	40.2	41.5	40.4

* The amount of conductivity was such that it was only just possible to make settings.

troduced into the cell and measured the results showed very conveniently that up to conductivities so great that it was only just possible to make settings the observed value of the dielectric constant was actually independent of conductivity. The figures obtained in such a calibration are given in Table II, which makes it quite clear that no corrections for conductivity and absorption are required for the values of the dielectric constant (ϵ') obtained with this procedure.

IV

By means of the first method measurements were made on solutions of zein of different concentrations in 70 per cent *n*-propyl alcohol. The temperature was varied between 0° and 80°. Owing to the nature of the method, the frequency changed accord-

ing to the value of the dielectric constant measured, but lay always between 3.8 and 4.7 meters.   In Fig. 3 the values of the dielectric constant obtained in this way are plotted against temperature.   At the lower temperatures these results show the same kind of effect that was observed by Fürth in the case of aqueous solutions of gelatin and albumin; *i.e.*, the dielectric constant of the protein solutions is lower than that of the pure
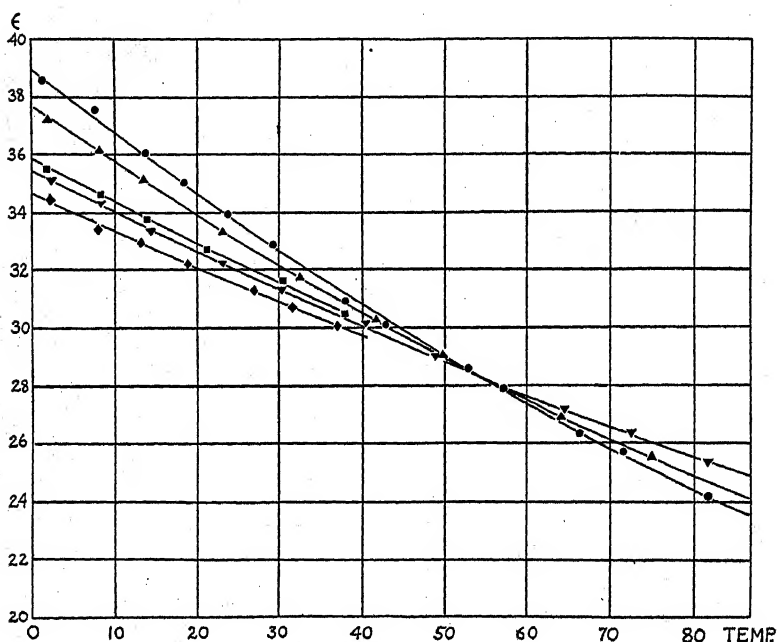
FIG. 3. The effect of temperature on the dielectric constant of solutions of zein dissolved in 70 per cent *n*-propyl alcohol measured at a wave-length of about 4 meters. The concentrations of the solutions are as follows: ●, 0.0 per cent; ▲, 4.1 per cent; ■, 9.9 per cent; ▼, 12.3 per cent; ◆, 14.2 per cent.

solvent by an amount increasing with the concentration of protein. As the temperature is raised, however, this effect diminishes and at the highest temperatures studied is actually reversed, the dissolved protein producing an *increase* of the dielectric constant.

Let us consider how these results may be explained in the light of the Debye theory.   In the first place it is important to be

clear in regard to the effect of temperature on the dielectric constant of the solvent itself. This is a mixture (solution) of two polar liquids, water and alcohol, in fixed proportions. Notwithstanding this polarity, however, we will apply the Mosotti relation in order to get a qualitative picture of the situation just as was done in the case of Mizushima's studies on glycerol and the alcohols. The dielectric constant is then given in terms of the molar polarizations of the two components by equation (9). These may of course vary with the composition of the solution, but in the present case this is constant. Since both the molecules are polar it follows from equation (10) that these polarizations will decrease with rise of temperature, owing of course, to the disturbing effect on orientation. At the same time there will also be a decrease of density accompanying an increase of temperature. Of necessity we neglect any influence of temperature due to changes of aggregation. By referring to equation (9) again it can be seen that the two effects, on the polarization and on the density, combine to produce a diminution in the dielectric constant. The general agreement between this picture and the experimental facts makes it clear that we are not concerned with any appreciable amount of absorption, for this would lead to just the opposite effect of temperature on the dielectric constant, as may be inferred *a priori* from the theory, and has been shown experimentally by Mizushima. Mizushima's results on propyl alcohol show, however, that at the lower temperatures we may not be very far above the region of dispersion.

But consider now the main phenomenon, the effect introduced by the zein. As in the case of Fürth's results the decreased dielectric constants encountered at the lower temperatures might be explained either in terms of a very small polarization of the zein molecules themselves or by a lowering of the polarizations of the alcohol and water produced by the protein, as if for instance the zein gave rise to a strong saturation effect by dominating the surrounding molecules of the solvent and preventing them from orienting. On the basis of either of these interpretations, however, it is very hard to see why the effect should be reversed by such a moderate rise of temperature. We should only expect such a reversal if we had reached the point where, owing to the temperature effect, the optical contributions to the dielectric

constant (unrelated to the permanent moments involved at the lower temperatures) had become predominant. But the optical value of the dielectric constant of most liquids is in the neighborhood of 2, whereas the phenomenon in question occurs at a temperature where the dielectric constant is still about 30.

A more reasonable interpretation is to assume that the zein is undergoing anomalous dispersion. This makes the situation at once easily intelligible. As the temperature is increased the relaxation time of the molecules is diminished (see equation (5)). Since the extent of the dispersion is determined by the value of the product of relaxation time and frequency (equation (13)) it follows directly that the effect of raising the temperature is the same as that of decreasing the frequency, as was shown experimentally by Mizushima. Accordingly we expect that at the lower temperatures, corresponding to high frequencies, the zein molecules can orient only slightly and contribute little more than their optical value to the dielectric constant. But as the temperature is increased the amount of orientation rises just as if the frequency were diminished at constant temperature and this, of course, results in an increase of the mean moment and a larger contribution to the dielectric constant. On the other hand, as we have seen, the effect of temperature on the solvent molecules leads to a diminution of the mean moment. Consequently, although at the lower temperatures the contribution of the zein molecules to the dielectric constant will be too small to compensate for the loss due to displacement of the solvent, at the higher temperatures it will more than make up for it. It ought to be pointed out in this connection that in view of the high viscosity of the zein solution as compared with the pure solvent it is quite probable that the alcohol molecules may themselves also begin to show dispersion at the lower temperatures. This of itself, however, would not be expected to account for the observed increase of dielectric constant though it would contribute to the decrease at the lower temperatures. On the basis of this explanation we might expect from equation (9) very large values for the molar polarization of the zein molecules, but this point will be dealt with later.

Plausible as it may be, the above interpretation of the behavior of zein must remain after all a hypothesis until supported by

further evidence. The measurements just discussed were all made at about the same frequency, the range being so small that the frequency can be regarded as approximately constant from the point of view of dispersion. What is plainly needed is a wider set of measurements in which frequency is varied as well as temperature. To obtain such data resort was had to the second method described above.

By means of this method the dielectric constants of zein solutions were determined at the five standard wave-lengths—260, 195, 152, 97.5, and 48.8 meters—and at different temperatures. These measurements were supplemented by determinations made by the earlier method at a wave-length of about 4 meters and at the same temperatures so as to obtain values at six different frequencies in all. They were made on zein at two concentrations in 70 per cent n-propyl alcohol; 0.105 and 0.042 gm. per cc. at 25°. The mol fractions of the three components of these two solutions were respectively (assuming a molecular weight of 100,000 for zein): zein, 5.52×10^{-5} and 2.1×10^{-5}; alcohol, 3.5 and 3.5; water 6.5 and 6.5.[4] The temperatures fixed for the measurements were 0°, 25°, and 50°; in the case of the more dilute solution additional measurements were made at 69.5°. The results of the study are given in Tables III and IV. In addition values of $\frac{\epsilon}{\epsilon_0}$, the ratio of the dielectric constant of the solution to that of the pure solvent, determined from the data given in Tables III and IV are plotted against wave-length in Figs. 4 and 5.

These results extended to longer wave-lengths show up clearly the effect which was only incipient in the earlier work at 4 meters, namely the increase in the dielectric constant resulting from addition of zein to the solvent. It is now seen that this increase may be very considerable. From Fig. 5 it appears that in the case of a 10.5 per cent solution of zein at 50° it amounts to over 50 per cent at a wave-length of 195 meters, and the shape of the curve suggests even higher values at still longer wave-lengths. That this increase is due to anomalous dispersion now appears indubitable, for the various confusing possibilities involved in the earlier

[4] The mol fractions of the zein are too small to effect the values for the other two components.

results at a single wave-length can all be ruled out by considering a fixed temperature and variable frequency. Here there can be no question of the effect of temperature on the values of the polarizations or on the interactions of the molecules, and the only way in which the dielectric constant can vary with frequency is owing to anomalous dispersion. A possible objection that at the higher

TABLE III

Effect of Wave-Length (λ) on the Dielectric Constant of a 10.5 Per Cent Solution of Zein in 70 Per Cent n-Propyl Alcohol at Various Temperatures

ϵ_0 = dielectric constant of the solvent.

λ	1–2°, ϵ_0 = 39.2	25.4°, ϵ_0 = 33.9	50°, ϵ_0 = 29.3
meters			
260	41.2	43.6	
195	40.3	42.2	45.0
152	39.5	40.8	42.6
97.5	38.3	38.6	39.9
48.8	36.9	35.2	33.5
About 4.3	35.2	31.9	28.8

TABLE IV

Effect of Wave-Length (λ) on the Dielectric Constant of a 4.2 Per Cent Solution of Zein in 70 Per Cent n-Propyl Alcohol at Various Temperatures

ϵ_0 = dielectric constant of the solvent.

λ	0.5°, ϵ_0 = 39.4	25.5°, ϵ_0 = 33.9	50°, ϵ_0 = 29.3	69.5°, ϵ_0 = 26.3
meters				
260	40.5	39.8	38.9	39.4
195	40.1	39.4	37.3	38.6
152	39.6	38.2	36.3	37.6
97.5	39.2	36.8	35.3	35.9
48.8	38.4	35.0	32.2	30.8
About 4.3	38.0	33.2	29.3	27.0

temperatures we may be dealing with effects resulting from denatured material is removed by the fact already pointed out in Section III that when the solutions are returned to the lower temperatures there is no measurable change in the properties studied. The large increase of the dielectric constant over the value for the pure solvent makes it evident that this dispersion involves primarily the protein molecules. Here again it must

not be overlooked that owing to the high viscosity of the zein solutions it is not improbable that at the shorter wave-lengths and lower temperatures the alcohol molecules may themselves begin to undergo dispersion. As before the result of this behavior on the part of the alcohol would be to augment the effect of diminished dielectric constants observed at the lower temperatures and higher frequencies; it could not, however, be expected to account
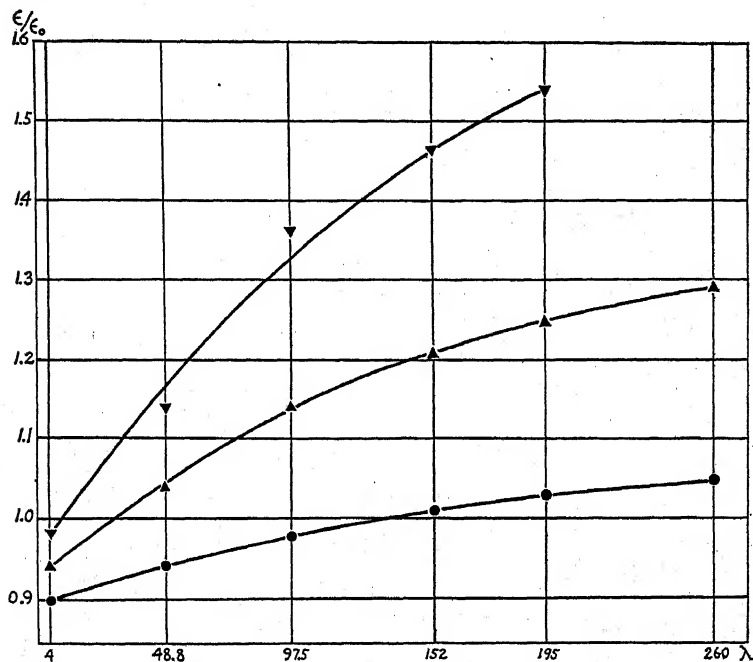
FIG. 4. Effect of wave-length (λ) on values of $\dfrac{\epsilon}{\epsilon_0}$ at various temperatures, taken from Table III.   ●, 1–2°; ▲, 25.4°; ▼, 50°.

for the large increase of dielectric constant observed at higher temperatures and lower frequencies. It may be remarked that the reciprocal effects of increasing the temperature and lowering the frequency characteristic of the dispersion range are illustrated by these results; for example, it is apparent from Figs. 4 and 5 that the frequency for which $\dfrac{\epsilon}{\epsilon_0} = 1$ increases as the temperature is diminished.

The existence of anomalous dispersion at these long wave-lengths in the case of zein arises from the great size of the molecule and has nothing to do with its specific chemical properties. We may expect, therefore, the phenomenon to be typical of the proteins as a class. This would explain at once the low values of the dielectric constants of gelatin and albumin observed by Fürth, for this observer was working at a very high frequency ($\lambda = 76$
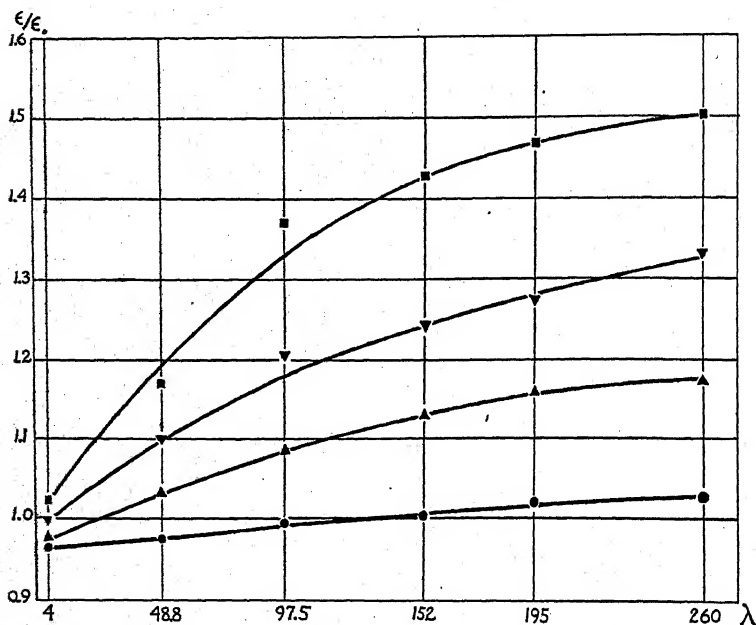
FIG. 5. Effect of wave-ength (λ) on values of $\frac{\epsilon}{\epsilon_0}$ at various temperatures, taken from Table IV.   ●, 0.5°; ▲, 25.5°; ▼, 50°; ■, 69.5°.

cm.) and was certainly below the region of dispersion. There is no reason to expect small moments for these proteins as compared with zein; rather in fact the contrary on the basis of the greater number of free acid and basic groups which they contain and their high valence type as indicated by their behavior in regard to solubility.[5]

The proteins are often regarded as colloids. In respect to this

[5] See Cohn (4) and later papers in *J. Gen. Physiol.*

point it is worth calling attention to the fact that most colloidal solutions studied do not show dielectric constants appreciably different from that of the dispersion medium. On the other hand Errera (13) has shown that solutions of venadium pentoxide (dispersed in water) may have enormously high dielectric constants, in fact reaching values as great as 1200, although this phenomenon was only observed after the solutions had been allowed to stand for some time. This standing was supposed to allow the growth of submicroscopic needle-shaped crystals. Errera also reported an increase of dielectric constant with wave-length up to 260 kilometers, indicating anomalous dispersion as would be expected. No effort was made to interpret these results in terms of Debye's theory, though they certainly appear to demand the existence of enormous moments in the colloidal particles. It was pointed out by Errera that this behavior appears to be associated with the presence of double refraction in these solutions. It is suggestive to observe that similar phenomena in regard to double refraction were found in muscle globulin by von Muralt and Edsall (14).

<center>V</center>

There appears to be no question then of the qualitative meaning of these results on zein in terms of the Debye theory. It is worth while, however, in view of the speculations of Section II to pursue the matter further in order to obtain if possible some idea of the actual magnitude of the molecular constants of the zein molecules. For this purpose it is necessary to apply the relations presented in Section I to the case where the approximations can no longer be regarded as strictly valid. The trouble, of course, all reduces ultimately to the assumption underlying the Mosotti relation. Nevertheless, the extremely good agreement between theory and experiment obtained in the case of Mizushima's results on glycerol and the alcohols, all highly polar liquids, is encouraging, and suggests that we may at least hope to obtain results of the right order of magnitude by applying the theory to the present problem.

Since we are concerned with solutions we will base our considerations on equation (9′) (as being somewhat simpler to apply in the present case than the equivalent equation (9)) and denote the three components, alcohol, water, and zein, by the subscripts 1, 2,

and 3 respectively. Let us now consider the polarization of the zein molecules, P_3. This will consist of two parts, an optical part $\dfrac{4\pi N \alpha_0}{3}$ independent of frequency and a complex part arising from orientation given by

$$4\pi N \; \frac{\mu^2}{3\, KT\, (1 + i\,\omega\,\tau)}$$

(see equation (11)). For convenience we denote these by P_3' and P_3'' respectively. We can then write equation (9') as follows:

$$(14) \qquad \frac{\epsilon - 1}{\epsilon + 2} = \left(c_1 \frac{P_1}{M_1} + c_2 \frac{P_2}{M_2} + c_3 \frac{P_3'}{M_3} \right) + c_3 \frac{P_3''}{M_3}$$

If we are working in a range in which only the zein molecules show absorption the expression in the parenthesis, which we will call P_0, will be constant for any given temperature and composition of the solution. On the other hand the complex term $\dfrac{c_3 P_3''}{M_3}$ will vary, passing towards its static value at the longer wave-lengths and approaching zero at the shorter wave-lengths. Within the range in question the dielectric constant will therefore be complex and its real part ϵ' (actually the observed dielectric constant) will pass from a lower value ϵ_0 to an upper value ϵ_1, just as if we were dealing with a single substance, according to equation (13); at the same time the imaginary part ϵ'', indicating absorption, will rise from zero to a maximum and then again become inappreciable, (also according to equation (13)). The effect of $\dfrac{c_1 P_1}{M_1}$ and $\dfrac{c_2 P_2}{M_2}$ is simply to increase the apparent optical value of the polarization of the zein molecules.

On the assumption that only the zein molecules take part in the dispersion it ought to be possible therefore to fit the experimental results at any temperature and concentration with the expression for ϵ' given by equation (13). This expression contains three constants, ϵ_0, ϵ_1, and τ, which can be determined from any three experimental points. The question, however, arises whether we really are justified in neglecting the possibility of dispersion on the part of the alcohol molecules. Owing to the high viscosity of the

protein solutions it appears likely that dispersion may occur, at least at the lower temperatures. If we apply Arrhenius' formula to interpolate the viscosities of the two zein solutions under consideration from the data given in Table I we obtain the following results.

Temperature..	25°	50°
Solution 1 (0.042 gm. per cc. at 25°)............	0.075	0.034
" 2 (0.105 " " " " 25°)..........	0.305	0.111

If we now turn to the data on pure n-propyl alcohol given by Debye ((1) p. 101) we find that at a wave-length of 3.08 meters absorption is considerable at 20°, where the viscosity is 0.023; at 9.5 meters it is just beginning at the same temperature; at 50 meters it does not appear until $-40°$, where the viscosity is 0.154. This indicates that our assumption about the alcohol molecules may be valid in the case of Solution 2 if we consider only the observations at 50° and at wave-lengths of 48.8 meters and greater; and in the case of Solution 1 if we limit ourselves to the observations at 50° and 69.5° and to those results obtained at 25° down to $\lambda = 48.8$ meters. In order to be on the safe side however, we will consider only the results on Solution 2 at 50° and 69.5°.

If we now proceed to apply equation (13) to this material we obtain the following figures for the three constants:

A. Temperature = 69.5°; $\lambda = 3.88-260$ meters; $\eta = 0.017$.
$\qquad \epsilon_1 = 40.5 \qquad \epsilon_0 = 27.0 \qquad \tau = 2.8 \times 10^{-8}$ sec.
B. Temperature = 50°; $\lambda = 48.8-260$ meters; $\eta = 0.034$.
$\qquad \epsilon_1 = 41.0 \qquad \epsilon_0 = 30.8 \qquad \tau = 5.4 \times 10^{-8}$ sec.

The values of ϵ' computed from equation (13) with these constants lie on the corresponding smooth curves given in Fig. 5 showing that the observed points are fitted to an accuracy well within the limit of the experimental error.

From these results we can now calculate the permanent moment of the zein molecules. In order to do this it is first necessary to determine the static values of P_3''. From equation (14) and the discussion following it can be shown that for any pair of values ϵ_1 and ϵ_0 the corresponding static value of P_3'' is given by:

$$c_3 \frac{P_3''}{M_3} = \frac{\epsilon_1 - 1}{\epsilon_1 + 2} - \frac{\epsilon_0 - 1}{\epsilon_0 + 2}$$

M_3, the molecular weight of zein, is taken as 100,000. Values of c_3 (concentration in gm. per cc.) may be determined for the higher temperatures from the values at 25° by taking account of the densities; the latter may be interpolated from the data given in Table I. On this basis the two pairs of values of ϵ_1 and ϵ_0 derived above are found to yield the following values of P_3''.

Solution 2 at 69.5°.............................. P_3'' 81,000 cc.
    "   2 " 50°................................. P_3'' 51,000 " [6]

From these two static values of P_3'' we obtain finally by means of equation (10) the corresponding values of the permanent electrostatic moment of the zein molecules, *viz.* 67 and 52×10^{-18} E.S.U. respectively.

As was pointed out at the beginning of this section, the numerical values obtained by the above application of the Debye theory can only be regarded as very rough approximations although they ought to be of the right order of magnitude. It is reassuring indeed to reflect that since P_3'' is proportional to the square of the permanent moment μ the errors in determining the former quantity will be greatly reduced in the final result for the electric moment; *e.g.*, a twofold error in P_3'' will correspond to only a 40 per cent error in μ. But let us consider the results we have just obtained in connection with speculations of Section II.

We take up first the question of relaxation time. It was calculated in Section II on the basis of Stokes' formula that the relaxation time of a spherical molecule of the size of zein (gram-molecular weight 100,000) in a medium of viscosity 0.01 ought to be 8×10^{-8} sec. The relaxation times, 2.8×10^{-8} and 5.4×10^{-8}, just computed by the Debye theory from the experimental results both correspond to viscosities considerably greater than this, but since relaxation time and viscosity are proportional to one another they may be reduced for comparison. By multiplying 2.8×10^{-8} by $\dfrac{0.010}{0.017}$ and 5.4×10^{-8} by $\dfrac{0.010}{0.034}$ (the fractions being the appropriate viscosity ratios) we obtain respectively 1.7×10^{-8} and 1.6×10^{-8}. Both these values are about $\frac{1}{5}$ of that calculated in

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[6] It may be pointed out that molar polarization has the dimension of volume.

Section II, though it is important to remark that equation (13) is relatively insensitive to changes in values of τ.[7] Whether or not this discrepancy is due to the approximations involved in a quantitative application of the Debye theory or to inadequacy of Stokes' formula cannot be decided, though it is worth recalling that relaxation times calculated from the formula are in good agreement with the experimental results in the case of the data for n-propyl alcohol discussed by Debye. In any case, however, it is evident, regardless of the discrepancy, that these results are of quite another order of magnitude from those observed for most other molecules. The relaxation time of a water molecule at room temperature (viscosity 0.01) appears to be about 2.5×10^{-11} sec. while that of a molecule of n-propyl alcohol at the same temperature reduced to the same viscosity will be approximately 4×10^{-11}. Both these differ from the values in question for zein by a factor of 500 or 1000.

Owing to the nature of the analysis we should expect that if the relaxation times obtained by fitting experimental data were too small the corresponding values of P_3'', and accordingly also of μ, though to a much less extent, would be so likewise.[7] But at all events the values of the permanent electrostatic moments obtained indicate that the zein molecules are highly polar. The average of the two values of the moment computed above is 60×10^{-18} E.S.U. It is interesting to compare this with a number of moments selected from those listed by Debye ((1) pp. 40–50).

Nitrobenzene	dissolved in benzene	3.90×10^{-18}
p-Dinitrobenzene " " "		0.32×10^{-18}
o-Dinitrobenzene " " "		6.05×10^{-18}
Iodine " " "		1.4×10^{-18}
Ethyl alcohol in carbon tetrachloride		1.63×10^{-18}
" " (gaseous state)		1.1×10^{-18}

The moment 6.05×10^{-18} for o-dinitrobenzene, although much the largest in Debye's list, is only about one-tenth of what we have estimated for zein.

[7] For example, even if we double the value of τ just determined for 69.5° the experimental points are still fitted to about 5 per cent provided we change ϵ_1 to 48 and ϵ_0 to 29. This would only correspond to a change of μ from 66×10^{-18} to 69×10^{-18} E. S. U.

The value 60×10^{-18} is very close to the figure 72×10^{-18} E.S.U. arrived at in Section II on the basis of two elementary charges, one positive and one negative, separated by a distance equal to half the radius of a zein molecule (assumed spherical), although of course such good agreement must be regarded as wholly fortuitous and cannot be taken as a justification of any such simple model.   Indeed if we accept the *Zwitter Ion* picture it is far more reasonable to suppose that the moment arises as the net result of a much larger number of pairs of elementary charges derived from the internal neutralization of the free acid and basic groups and correspondingly distributed in the molecule.   As already pointed out (Section II), the number of such free basic groups in the zein molecule appears to be sixteen and that of free acid groups thirty.   On the basis of complete internal neutralization we might expect, therefore, a maximum number of sixteen pairs of charges contributing to the net moment.   There is undoubtedly much evidence in favor of the existence of proteins in the *Zwitter Ion* form.[8]   On the other hand, we ought not to ignore the effect of the large number of polar groups contained in the zein molecule.   A picture of this may be obtained from a consideration of the amino acid content given by Cohn (5).   The contributions of all these polar groups will add vectorially, but whether they will largely neutralize one another as in the case of the NO_2 groups in p-dinitrobenzene or supplement one another as in the case of the ortho compound cannot be predicted.   The present conclusions as to the resultant moments of the zein molecules do not help us to settle the point.

The present results have some interest in connection with recent work on the raising of body temperature and production of fever by high frequency power oscillators.   It was originally observed by Dr. W. R. Whitney of the research laboratories of the General Electric Company that individuals working in the field of a high frequency radio transmitter showed a rise of body temperature. Since then Carpenter and Page (17), also of the General Electric Company, have produced increases of temperature amounting to about $3°$ in little more than an hour on patients placed between the

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[8] Evidence bearing on this point is discussed by Borsook and MacFayden (15), and Weber (16).

plates of a large condenser forming part of an oscillatory circuit operating with two 500 watt radiotrons.[9] These results are explained by Carpenter and Page on the basis of the conductivity of the protoplasm. Similar heating effects can be obtained if salt solutions are introduced between the plates of the condenser, and indeed it can be shown theoretically that this will be the case and that the liberation of heat will be a maximum for a certain conductivity.[10] The present work on anomalous dispersion suggests that this picture may be incomplete and that a rotational absorption by the body proteins will also play a part in the development of heat and ought to be taken into account. This will give rise to exactly the same effects as absorption due to conductivity but will represent a different process and will be differently related to frequency. Whereas the conductivity effect will increase linearly with the wave-length the rotational effect will pass through a maximum at a frequency close to that given by the relaxation time of the molecules (see Section I). But although we may be sure that it must play a part in the heat production, it is impossible to say how important this rotational absorption will be in comparison with the effects of conductivity except on the basis of more extended data.

VI

As a conclusion to this study on zein an investigation was made of the gels which form when solutions of the protein are allowed to stand at room temperature. It seems probable that this gelation is the expression of a denaturation or alteration of the molecules; but quite apart from this question it results, of course, in a tremendous rise of viscosity. This alone would lead us to expect a change in the position of the anomalous dispersion. In order to examine this point a solution of zein (0.084 gm. per cc. at 25°) in 70 per cent n-propyl alcohol was introduced into the cell shown in Fig. 2 and measured at intervals until gelation had occurred. The temperature of all the measurements was 29° and the frequencies were those listed in terms of wave-length in Table V. On June 18 (the day when the solution was made up)

[9] The most effective wave-length was found to be 30 meters.
[10] An unpublished result given by G. W. Pierce.

and June 19 there was no sign of gelation; on June 21 the solution had become markedly more viscous, though it was still fluent; on June 25 gelation was complete. The data show that over this whole period there was no measurable change in the apparent dielectric constant at any of the frequencies, and consequently no shift in the anomalous dispersion. This result is certainly surprizing in view of the tremendous viscosity of the gels, for we should expect from this a very great increase in the relaxation time of the molecules accompanying gelation. The only interpretation of the absence of any such effect seems to be that gelation has nothing to do with the real viscosity of the solutions concerned in Stokes' formula and related to relaxation times. We might suppose that the gel results from the formation in the solution of something like a network of a mesh large in comparison with molecular dimensions though invisible from the microscopic point

TABLE V

Dielectric Constant of Zein Gels (See Text) at Various Wave-Lengths (λ) in Meters

Date	$\lambda = 260$	$\lambda = 195$	$\lambda = 152$	$\lambda = 97.5$	$\lambda = 43.8$
June 18................	42.1	40.6	39.2	38.5	35.4
" 19................	42.3	41.1	39.5	38.4	35.9
" 21................	42.1	40.7	39.6	38.3	35.2
" 25................	42.3	41.0	39.6	38.2	35.4

of view, and that only a small fraction of the molecules are involved in the network (for otherwise the dielectric constant would be lowered). Such a conclusion is suggested also by the elastic behavior of gels and is in agreement with observations on the migration velocity of ions, which though under ordinary conditions obeying Stokes' law yet remain unaffected by gelation of the medium.

The writer wishes to express his thanks to Professor G. W. Pierce for this kindness in allowing him to carry out this work in the Cruft Laboratory. He is also grateful to Professor E. J. Cohn for many helpful suggestions in the writing of this paper.

SUMMARY

In order to study the dielectric constant of proteins in the light of Debye's theory two methods were developed based on reso-

nance.   By means of these methods measurements were made on
solutions of the protein zein dissolved in 70 per cent *n*-propyl
alcohol.

The results obtained at temperatures ranging from 0° to 80°
and wave-lengths from 4 to 260 meters, show the presence of
anomalous dispersion and indicate that the zein molecules are
highly polar.

A quantitative application of Debye's theory leads to a value
of about 60×10^{-18} E.S.U. for the permanent electric moment of
the molecules, and to values for the relaxation time somewhat
smaller than those calculated on the basis of Stokes' formula for
the rotation of a spherical particle in a viscous medium.   These
numerical values are only roughly approximate however.

An extension of the measurements to gels which form when the
solutions of zein are allowed to stand showed no change in dielec-
tric constant accompanying gelation.   This suggests that the
viscosity of the gels is not the ordinary viscosity entering Stokes'
expression.

List of Symbols

D, electric displacement
ϵ, dielectric constant
E, electric intensity
I, electric moment per unit volume
n, number of molecules per cc.
m, total moment per molecule
m_0, induced moment per molecule
α_0, optical polarizability
α, constant defining total moment assumed by the molecules
a, molecular radius
F, electric force
μ, permanent electric moment of a molecule
$\overline{m}$, mean moment per molecule due to orientation
K, constant $= 1.37 \times 10^{-16}$
T, absolute temperature
t, time
N, Avogadro's number $= 6.06 \times 10^{-23}$
i, $\sqrt{-1}$
e, base of natural logarithms
η, viscosity
ρ, density
τ, relaxation time
ζ, frictional resistance

P, polarization
f, mol fraction
c, concentration in gm. per cc.
ω, $2\pi \times$ frequency
λ, wave-length
Λ, specific conductivity

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URSOLIC ACID*

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In a former communication (1) the writer reported the results of an investigation of the constituents of the wax-like coating on the surface of the apple (Ben Davis and Black Ben Davis), from which there was isolated among other substances, a crystalline compound melting at 284–285° which was named malol. In addition to studying the carefully purified compound a number of derivatives were prepared and analyzed, including the monosodium salt, the diacetyl and the monoacetyl derivatives, the monomethyl, and the monoacetylmonomethyl derivatives. The combustion results of the parent substance and of the derivatives mentioned above indicated very strongly that the true formula of malol was $C_{30}H_{48}O_3$.

While it was pointed out that malol possessed the same formula and properties as urson, originally isolated by Trommsdorff (2) from the leaves of *Arctostaphylos uva-ursi*, and also exhibited the same general properties as prunol, isolated from the leaves of the wild black cherry, *Prunus serotina*, by Power and Moore (3), the three substances were not considered identical since none of the melting points reported for urson or prunol and their derivatives agreed with those found for malol. It was realized, however, that the statements in the literature relating to urson were very contradictory, and mention was made that further investigation of this product was needed.

Nearly 1 year later (1924), van der Haar (4) published the results of a reinvestigation of urson. He assigned to it the formula $C_{31}H_{50}O_3$ and concluded that it is a hydroxy acid, thus

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* Contribution No. 96 from the Food Research Division.

confirming Nooijen's (5) contention that the substance contains a
carboxyl group. In accord with its chemical character he sug-
gested the name ursolic acid. On the basis of this work, van der
Haar concluded, in later publications (6, 7), that malol, prunol,
and urson are identical and that the formula reported for malol
should, therefore, conform with that claimed by him for ursolic
acid, or $C_{31}H_{50}O_3$ instead of $C_{30}H_{48}O_3$.

He (6) made the following statements in this connection.

"Nun hat aber Sando für sein Malol die Formel $C_{30}H_{48}O_3$ angegeben.
Ich habe ausführlich dargelegt, dass die Formel für Urson $C_{31}H_{50}O_3$ ist.
. . . Sando hat zweifellos zu niedrige C-H-Zahlen erhalten, und ich
möchte ihn auffordern seine Substanz in Mengen von 100-120 mg noch
einmal mit Kupferoxyd im Sauerstoffstrome und mit Kupferoxydpulver im
Schiffchen sehr langsam zu verbrennen."

It is interesting to note that Gintl (8), the first to make a
critical study of urson, assigned to it the formula $C_{30}H_{48}O_3$, and
Dodge (9) in 1918 and Nooijen (5) in 1920 reported results which
led them to accept and support the Gintl formula. Furthermore,
Rivière and Pichard (10) in 1925, working with malol from apples,
also accepted the $C_{30}H_{48}O_3$ formula although at the time they were
undoubtedly unaware of the possible identity of malol with urson.

More recently, 1930, Dodge (11) in reply to van der Haar says:
"To sum up, the question as to the elementary composition of
these compounds[1] may be considered as open, with the evidence
rather in favor of the C_{30} formula."

It is true that Power and Moore (3) assigned the formula
$C_{31}H_{50}O_3$ to prunol which is claimed by van der Haar to be identi-
cal with urson, but the results of their analysis indicate just as
conclusively the C_{30} formula. On the basis of their actual figures
van der Haar (6) is not justified in claiming this work as a con-
firmation of his C_{31} formula.

In the paper by van der Haar (7) to which reference has been
made, he also refused to accept the writer's claim that malol is
capable of yielding a diacetyl derivative which in turn is converted
into a monoacetyl derivative by heating with alcohol. He says:

"Sando behauptet ein Diacetylmalol mit dem Schmelzpunkte 199-200°
dargestellt zu haben. Dieser Schmelzpunkt stimmt aber völlig mit dem

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[1] Dodge here refers to caryophyllin and urson.

von mir für Monoacetylurson (= Monoacetylprunol) gefundenen überein. In vorhergehenden Abhandlungen . . . habe ich schon ausführlich erörtert, dass bei der Acetylierung kein Diacetylurson gebildet wird, sondern ein Monoacetylurson mit dem Schmelzpunkte 200–201°. Sando hat zweifellos ein Monoacetylmalol dargestellt. Sando erhitzt sein vermeintliches Diacetylmalol mit Alkohol und will daraus ein Monoacetylmalol erhalten haben. Aus dem erhaltenen Schmelzpunkte 279–281° aber hätte Sando ersehen können, dass er aus dem zuerst entstandenen Monoacetylmalol sein ursprüngliches Malol zurück erhalten hatte."

In referring to van der Haar's original work on urson (4) and to his later criticism of Power and Moore's work on prunol (6), the writer finds equally positive statements to the effect that a diacetyl derivative could not be formed by heating the parent substance with acetic anhydride. Furthermore, van der Haar also refused to accept the conclusion of Dodge working with urson, and of Power and Moore working with prunol, that heating the alleged diacetyl compound with alcohol gives rise to a mono-acetyl derivative.

In a more recent article, however, van der Haar (12), with a preponderance of evidence against him and as a result of more careful and painstaking work, completely revises his previous conceptions and admits that ursolic acid yields a diacetyl compound which may be converted into a monoacetyl derivative by boiling with alcohol. He now claims that the melting point of the monoacetyl compound was in close agreement with that of pure ursolic acid. It was this similarity in melting point between the parent substance and its derivative that misled him. He failed to make an analysis of the substance which he supposed to be regenerated urson and thus arrived at an erroneous conclusion.

In view of the doubt, therefore, which appears to exist as to the true formula of ursolic acid and the alleged formation of a diacetyl derivative it seemed desirable to pursue the investigation of the substance with the hope that additional light would be thrown on these points. The results of a further study are embodied in this paper.

<div align="center">EXPERIMENTAL</div>

Isolation of Urson—The material employed for the isolation of urson (ursolic acid) consisted of the leaves of *Arctostaphylos uva-ursi*, commonly known as bearberry. This was kindly

supplied by S. B. Penick and Company.   As received, it consisted
of the coarsely ground, air-dried leaves.   The material, ground
finer in a drug mill, weighed approximately 2200 gm.   It was first
extracted in a percolator with successive quantities of cold petro-
leum ether (b.p. 50–70°), to free it of "lipides" and coloring matters,
and finally with U.S.P. ether in a continuous extractor of the
Soxhlet type.   The solvent soon became supersaturated and
deposited in the receiving flask crude urson which was removed by
filtration from time to time to prevent bumping.   This material
together with the darker residue obtained by the complete evapora-
tion of the solvent was dissolved in boiling alcohol containing
about 1 per cent sodium hydroxide.   A trace of insoluble matter
was removed and an equal quantity of hot water added to the
filtrate.   Upon evaporation of most of the alcohol a copious
separation of the semi-crystalline sodium salt occurred.   The
precipitate was collected by suction and dissolved, without drying,
in cold 95 per cent alcohol containing a little sodium hydroxide.
The solution was filtered and after the addition of water, the
alcohol evaporated until crystallization occurred.   The foregoing
process was repeated until the sodium salt became nearly snow-
white, when it was dried and extracted with ether to remove
impurities.   The fairly pure sodium salt was then dissolved in
warm alcohol, and hot dilute aqueous hydrochloric acid slowly
added with stirring, whereupon amorphous urson separated.
This was collected on a Buchner funnel, washed well with hot
water, and dried.   The total quantity of urson thus obtained
amounted to approximately 30 gm.   It was boiled with one-fifth
the total quantity of 75 per cent alcohol necessary to dissolve
the whole.   The remaining insoluble portion was then heated
with successive quantities of the same concentration of alcohol
until in all five fractions were obtained.   In each case the hot
filtered solution deposited crystalline urson on cooling.   A deter-
mination of the melting point of each fraction indicated that the
first separation contained most of the impurities.   This portion
was, therefore, discarded and the remaining four fractions com-
bined and recrystallized from 95 per cent alcohol until the melting
point remained constant.

    The method of purifying urson just described is essentially the
same as that employed by Power and Moore (3) for the preparation

of prunol, the differences being that the partially purified sodium salt was freed of possible traces of alcohols and hydrocarbons by extraction with ether and in addition the regenerated parent substance was fractionally crystallized from alcohol to eliminate the fraction having the least purity. These added precautions possibly may account for the fact that a more homogeneous and purer product, as judged by the melting point, was ultimately obtained.

Urson crystallized in colorless, highly lustrous prismatic needles which, when placed in the bath at 270° and heated slowly, melted at 284–285°,[2] without apparent decomposition. This ·melting point is in perfect agreement with that previously reported (1) for malol isolated from apple skins and for the sample of urson studied by Dodge (9), but fails to agree with the melting point reported for urson by van der Haar (4). Taken in Roth's apparatus, he found this to be 279–280°. Whether or not the lower melting point is an indication of the presence of an impurity is not definitely known, but this fact taken in conjunction with his combustion data would seem to suggest the possible presence of a trace of hydrocarbon. Such an impurity would tend to lower the melting point and give rise to slightly higher carbon and hydrogen results.

Since theoretical carbon and hydrogen percentages representing the C_{31} or van der Haar formula show but slight variations from the corresponding values for the C_{30} formula it seemed desirable and necessary to make more than the usual number of combustions and to average the final results[3] in order to. arrive at a more accurate conclusion.

The results[4] of the combustions[5] of the compound, dried to constant weight at 160–165°, are as follows:

[2] All melting point determinations made in the course of this investigation were obtained by the use of Wheeler total immersion thermometers which were standardized by the United States Bureau of Standards.

[3] However, only those combustions are reported which show minimal and maximal carbon percentages. The mean values for carbon and hydrogen have been obtained by averaging the results from the total number of determinations which are indicated in parentheses.

[4] In the subsequent calculations H = 1.008 is used.

[5] Van der Haar (7) raises some questions in connection with the writer's previous combustion results on malol. He claims carbon and hydrogen

0.1038 gm.: 0.2988 gm. CO_2 and 0.0965 gm. H_2O. Found. C 78.51, H 10.40.

0.0949 gm.: 0.2746 gm. CO_2 and 0.0917 gm. H_2O. Found. C 78.92, H 10.81.

Found, mean value (10 combustions). C 78.77, H 10.56.

$C_{30}H_{48}O_3$ requires C 78.88, H 10.60.

$C_{31}H_{50}O_3$ "   " 79.08, " 10.71.

Isolation of Prunol—The material employed for the isolation of prunol was from the same source as that used by Power and Moore (3); namely, the leaves of the wild black cherry, *Prunus serotina*, Ehrhart. These were collected in August, near Washington, D. C. They were air-dried, ground, and the prunol extracted therefrom in the same manner as described under the isolation of urson.

The pure crystals of prunol possessed the same appearance and practically the same melting point, 285–285.5°, as urson.

Combustions[6] of the anhydrous substance, dried at 160–165°, yielded the following results.[3]

0.1085 gm.: 0.3127 gm. CO_2 and 0.1026 gm. H_2O. Found. C 78.60, H 10.58.

0.0952 gm.: 0.2754 gm. CO_2 and 0.0903 gm. H_2O. Found. C 78.90, H 10.61.

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results were too low and that the substance should be mixed with powdered cupric oxide and burned slowly with oxygen. It seemed desirable, therefore, briefly to mention the apparatus used in this study and some of the precautions taken. Oxygen, 99.5 per cent purity, was employed as in the previous investigation (1) and all analyses were made in a multiple unit electric furnace equipped with a preheated tube containing cerium dioxide and a mixture of copper oxide and lead chromate. The drying train consisted of sodium hydroxide, sulfuric acid, and calcium chloride. The combustion gases were collected in a calcium chloride U-tube and a Gomberg potash bulb which were weighed very carefully by the counterpoise method with platinum-plated weights standardized by the United States Bureau of Standards. Several combustion tube fillings were employed. In the case of data given under "Isolation of urson" copper oxide only was used. The substance was not mixed with powdered copper oxide. Palladious chloride solution at the absorption end of the train was included to indicate the absence of carbon monoxide.

[6] The combustion tube filling consisted of cerium dioxide and copper oxide. No powdered copper oxide was mixed with the substance. Palladious chloride solution at the absorption end of the train indicated the absence of carbon monoxide.

Found, mean value (10 combustions). C 78.79, H 10.67.
$C_{30}H_{48}O_3$ requires C 78.88, H 10.60.
$C_{31}H_{50}O_3$ "   " 79.08, " 10.71.

Isolation of Malol, Method I—The material employed for the isolation of malol consisted of the peels of the McIntosh apple obtained as a by-product in the investigations of Power and Chesnut (13) on the odorous constituents of apples. In their work, the fresh parings had been subjected to distillation in a current of steam. For the removal of malol, the air-dried residue was ground and extracted first with petroleum ether and then with U. S. P. ether. Crude malol was obtained upon evaporation of the ether extract. The method used for the purification of urson was employed in the case of malol. It was based on the purification of the sodium salt from which the parent substance was regenerated. The compound possessed the same crystal form and appearance as urson and prunol. The purest fraction, after recrystallization from 95 per cent alcohol, melted at 284–285°, thus agreeing with the melting point previously reported (1) for the same substance isolated from the mixed parings of Ben Davis and Black Ben Davis apples.

Combustions[7] of the anhydrous compound, dried to constant weight at 160°, yielded the following results.[3]

0.0985 gm.: 0.2836 gm. CO_2 and 0.0923 gm. H_2O. Found. C 78.52, H 10.48.
0.1043 gm.: 0.3021 gm. CO_2 and 0.0980 gm. H_2O. Found. C 78.99, H 10.51.
Found, mean value (9 combustions). C 78.79, H 10.58.
$C_{30}H_{48}O_3$ requires C 78.88, H 10.60.
$C_{31}H_{50}O_3$ "   " 79.08, " 10.71.

Isolation of Malol, Method II—The sample of malol considered under Method I was purified by conversion into its sodium salt. It was thought desirable to obtain a preparation by the use of neutral solvents only, thus avoiding the criticism that a chemical change possibly might have occurred in the substance through the use of alkali and acid.

[7] The combustion tube contained palladiumized asbestos, cerium dioxide, and copper oxide in the order given. The substance was mixed with powdered cupric oxide, and burned very slowly.

A portion of the original crude malol obtained from the ether extract of the dried peels was washed repeatedly with cold petroleum ether (b.p. 40–50°) and then with successive small quantities of ether to remove as much color and other impurities as possible. The residual ivory-white amorphous powder was crystallized from hot absolute alcohol and separated by the method described under "Isolation of urson" into six portions by fractional solubility in 70 per cent alcohol. Each filtrate on cooling deposited crystalline malol. The first fraction was discarded, and the remaining portions were combined and recrystallized from 95 per cent alcohol. In the process of crystallization a small quantity of amorphous, flaky matter separated on partial cooling. Later, glistening needles of malol appeared. Most of the flaky material was removed mechanically by carefully pouring off the liquid in which it became suspended on slight agitation. Most of the remaining flaky substance was removed by washing the residue with successive small portions of alcohol and pouring off the washings. The crystalline malol was finally recrystallized from 95 per cent alcohol whereupon it separated in beautiful glistening acicular crystals, some of which were 1 cm. in length. After drying in a vacuum at 105° the substance, when placed in the bath at 260° and heated slowly, melted at 285°.

The pure compound, dried to constant weight at 160–165°, gave the following combustion[8] results.[8]

0.1070 gm.: 0.3080 gm. CO_2 and 0.1002 gm. H_2O. Found. C 78.50, H 10.48.

0.1024 gm.: 0.2973 gm. CO_2 and 0.0973 gm. H_2O. Found. C 79.18, H 10.63.

Found, mean value (9 combustions). C 78.82, H 10.58.

$C_{30}H_{48}O_3$ requires C 78.88, H 10.60.

$C_{31}H_{50}O_3$ "   " 79.08, " 10.71.

Confirmation of Identity of Urson, Prunol, and Malol—It is evident from the foregoing analytical data and physical properties that urson, prunol, and malol are identical, a conclusion confirming van der Haar's findings (6, 7) in this respect. In view of the

[8] The combustion tube was filled with palladiumized asbestos, cerium dioxide, and copper oxide. The substance was mixed with powdered copper oxide.

excellent agreement of these results, complete identification required only the determination of the optical crystallographic properties. For these additional data, the writer is indebted to Dr. Edgar T. Wherry and G. L. Keenen. They submit the following.

Urson, prunol, and malol are difficult to study by the immersion method under the polarizing microscope. The surfaces of the crystals are of such character that on raising the microscope tube, light usually appears to move in both directions from the edges, so that exact matching of their refractive indices against immersion media is difficult. Within the limits of error of measurement, however, they all agree in possessing the following properties. The habit is characteristic, blades being present with some cleavage crosswise. These show the indices α, crosswise of blades, equal to 1.55, β, lengthwise, equal to 1.56, and γ, perpendicular to the blades and measurable only on rare fragments which happen to turn on edge, equal to 1.58, all $\pm$ 0.01 because of the difficulty in matching. Between crossed nicols, interference colors of the first and second orders are distinguishable. Elongation is positive and extinction parallel. In convergent polarized light, with crossed nicols, biaxial figures are shown with $2E = 130° \pm 10°$. The optic sign is positive. The crystal system is inferred to be rhombic, with the directon of the index β lying along the principal zone axis.

These optical data do not agree very well with those published for urson by Dodge (9) and credited to J. P. Wintringham, but in view of the difficulty of matching refractive indices his n value of 1.53 to 1.54 is perhaps as close to that of α here given, as can be expected. The value of $2V$ calculated from the indices is 72° and that of $2E$ is 133°, the first being decidedly larger than Wintringham's 22–28° (although this may have been V rather than $2V$) and the second agreeing with the observed $2E$.

Since urson, prunol, and malol are identical and it has been shown that the compound contains a carboxyl group, the suggestion that it be called ursolic acid is hereby adopted.

The true empirical composition of ursolic acid appears to be represented by $C_{30}H_{48}O_3$ and not $C_{31}H_{50}O_3$ as claimed by van der Haar (4). This conclusion is based on the results of the numerous foregoing analyses and on the data of other investigators which are listed in Table I for comparison.

Diacetyl Derivative of Ursolic Acid, $C_{30}H_{46}O_3(COCH_3)_2$—Ursolic acid obtained from each of the three sources was acetylated by boiling several hours with an excess of acetic anhydride. The greater portion of the reagent was then removed by evaporation and the clear solution allowed to stand in a desiccator over caustic

TABLE I

Carbon and Hydrogen Results Obtained by Various Investigators*

Substance	Source	Investigator	No. of combustions	Mean value for C	H
Urson	*Arctostaphylos uva-ursi*	Gintl, 1893 (8)	3	78.73	10.97
Prunol	*Prunus serotina*	Power and Moore, 1910 (3)	1	78.71	10.98
Urson	"Merck"	Van Itallie, 1918 (14)	1	79.03	10.63
"	*Calluna vulgaris*	Nooijen, 1920 (5)	2	78.78	10.33
"	*Erica tetralix*	"	2	78.84	10.56
"	*Vaccinium macrocarpum*	"	1 (?)	78.80	10.51
"	*Ilex aquifolium*	"	2	78.84	10.51
"	*Empetrum nigrum*	"	1 (?)	78.85	10.55
Malol	*Pyrus malus* (Ben Davis and Black Ben Davis)	Sando, 1923 (1)	2	78.75	10.54
"	*Pyrus malus* (McIntosh) Sample I	" this paper	9	78.79	10.58
"	Sample II	" " "	9	78.82	10.58
Prunol	*Prunus serotina*	" " "	10	78.79	10.67
Urson	*Arctostaphylos uva-ursi*	" " "	10	78.77	10.56

Found, weighted mean of 53 analyses. C 78.79, H 10.61.

$C_{30}H_{48}O_3$ requires C 78.88, H 10.60.

$C_{31}H_{50}O_3$ " " 79.08, " 10.71.

Van der Haar (4) found, mean of two analyses. C 79.32, H 11.02.

* The results given in this table as well as subsequent data have been recalculated from each author's original data, but on the basis of H = 1.008.

potash sticks. Glistening, colorless needles gradually were deposited. These were filtered off by suction, washed with a small quantity of acetic anhydride, and recrystallized from the same solvent. The product was finely ground in a mortar and kept for some time under reduced pressure over caustic potash sticks to

remove the last traces of retained acetic anhydride and moisture. In one experiment 9.9 gm. of pure ursolic acid yielded 10.9 gm. of acetylated product. Heating this substance 1 hour at 160° caused no further loss in weight, thus indicating that free acetic anhydride was completely eliminated by the method employed. The fact that constant weight was maintained under these conditions also proves the absence of decomposition at this temperature.

Combustions were made of the anhydrous compound, dried to constant weight at about 160°, with the following results.[3]

0.1050 gm.: 0.2901 gm. CO_2 and 0.0902 gm. H_2O. Found. C 75.35, H 9.61.

0.0998 gm.: 0.2769 gm. CO_2 and 0.0865 gm. H_2O. Found. C 75.67, H 9.70.

Found, mean value (12 combustions). C 75.51, H 9.69

$C_{30}H_{46}O_3(COCH_3)_2$ requires C 75.50, H 9.70.

$C_{31}H_{48}O_3(COCH_3)_2$ " " 75.75, " 9.82.

Combustions[3] were also made of the anhydrous compound dried over caustic potash sticks *in vacuo* and then over phosphorus pentoxide without subsequent heating.

0.1018 gm.: 0.2812 gm. CO_2 and 0.0870 gm. H_2O. Found. C 75.33, H 9.56.

0.1075 gm.: 0.2983 gm. CO_2 and 0.0932 gm. H_2O. Found. C 75.68, H 9.70.

Found, mean value (7 combustions). C 75.52, H 9.66.

$C_{30}H_{46}O_3(COCH_3)_2$ requires C 75.50, H 9.70.

The foregoing data are in close agreement with those previously reported for diacetylmalol and for diacetylprunol. The writer (1) found C = 75.48 and H = 9.67 as average values for diacetylmalol, while earlier, Power and Moore (3) obtained C = 75.56 and H = 9.73 for the same derivative under the synonym of diacetylprunol.

Monoacetylursolic Acid, $CH_3CO \cdot O \cdot C_{29}H_{46}COOH$—For the preparation of monoacetylursolic acid the diacetyl derivative was dissolved in 70 per cent alcohol and the solution boiled under a reflux condenser for 3 hours. On cooling, monoacetylursolic acid separated as fine needles which melted at 289–290°. A further quantity was obtained by partial evaporation of the mother liquor. In this manner, 4.56 gm. of the anhydrous diacetyl

derivative yielded 4 gm. of monoacetylursolic acid. The melting point of the recrystallized substance agreed with that reported by Power and Moore (3) for monoacetylprunol (monoacetyl-ursolic acid). The lower melting point previously reported (1) for monoacetylmalol was probably due to the presence of a small quantity of the unchanged diacetyl derivative from which it was derived.

The combustion results[3] obtained from monoacetylursolic acid, dried at 160°, are given below.

0.1058 gm.: 0.2979 gm. CO_2 and 0.0952 gm. H_2O. Found. C 76.79, H 10.07.

0.1047 gm.: 0.2963 gm. CO_2 and 0.0943 gm. H_2O. Found. C 77.18, H 10.08.

Found, mean value (11 combustions). C 76.96, H 10.06.

$CH_3CO \cdot O \cdot C_{29}H_{45} \cdot COOH$ requires C 77.05, H 10.11.

$CH_3CO \cdot O \cdot C_{30}H_{48} \cdot COOH$ "   " 77.28, " 10.23.

Previous results of the writer (1) for monoacetylmalol were C = 77.20 and H = 10.17. Gintl (8) found, as an average of two determinations, C = 77.03 and H = 10.18, while Power and Moore (3) report C = 77.01 and H = 10.43, and van der Haar (4), C = 77.15 and H = 9.99. Averaging the results obtained from the sixteen determinations, including the ones reported in this paper and those from the other investigators mentioned, we find the values for C = 77.00 and for H = 10.11. Those figures agree better with the C_{30} formula than with the C_{31} formula for ursolic acid.

Regenerated Ursolic Acid—Heating monoacetylursolic acid 1 hour with alcoholic caustic potash (150 cc. alcohol + 3.5 gm. of caustic potash) converted the compound into ursolic acid. The parent substance was recovered by precipitation with hot dilute aqueous hydrochloric acid and then recrystallized from 70 per cent alcohol.

Dried to constant weight at 160°, the regenerated substance yielded the following results[3] on analysis.

0.1111 gm.: 0.3207 gm. CO_2 and 0.1038 gm. H_2O. Found. C 78.72, H 10.45.

0.1054 gm.: 0.3050 gm. CO_2 and 0.0993 gm. H_2O. Found. C 78.92, H 10.54.

Found, mean value (6 combustions). C 78.82, H 10.54.

$C_{30}H_{48}O_3$ requires C 78.88. H 10.60.

Methylursolate, $HO \cdot C_{29}H_{46}COO(CH_3)$—A quantity of ursolic acid was dissolved in absolute methyl alcohol. The solution was made slightly alkaline with sodium hydroxide, and then boiled with an excess of dimethyl sulfate. After making the final reaction mixture alkaline by the addition of a further quantity of sodium hydroxide, it was poured into water, whereupon a separation of the methyl ester occurred. The insoluble material was collected, washed with water, and dissolved in ether. After shaking the ethereal solution with dilute alkali several times and finally with successive portions of water, the ether was evaporated to dryness. The residue was crystallized from 70 per cent alcohol and dried at 125°. It then melted at 170.5–171.5° and gave the following results upon analysis.

0.0990 gm.: 0.2872 gm. CO_2 and 0.0949 gm. H_2O.
0.1014　"　: 0.2941　"　"　"　0.0978　"　"
Found.　C 79.12, 79.10; H 10.73, 10.79.
$HO \cdot C_{29}H_{46}COO(CH_3)$ requires C 79.08, H 10.71.
$HO \cdot C_{30}H_{48}COO(CH_3)$　"　"　79.27, "　10.82.

Previous results for monomethylmalol (1) were C = 79.16, H = 10.61. Nooijen (5) found the average values of three determinations to be C = 79.50 and H = 10.63.

Monoacetylmethylursolate, $CH_3CO \cdot O \cdot C_{29}H_{46} \cdot COO(CH_3)$—Monoacetylmethylursolate was obtained by acetylating the methyl ester of ursolic acid. Crystallized from dilute alcohol and dried at 125°, the compound melted at 246–247°. On long continued boiling with alcohol the acetyl group is not split off, as evidenced by the fact that the melting point remains unchanged. Combustions of the substance yielded figures which agree with those calculated from theory.

0.1028 gm.: 0.2911 gm. CO_2 and 0.0967 gm. H_2O.
0.1008　"　: 0.2859　"　"　"　0.0939　"　"
0.1010　"　: 0.2862　"　"　"　0.0912　"　"
Found.　C 77.23, 77.35, 77.28; H 10.53, 10.42, 10.10.
$CH_3CO \cdot O \cdot C_{29}H_{46} \cdot COO(CH_3)$ requires C 77.28, H 10.23.
$CH_3CO \cdot O \cdot C_{30}H_{48} \cdot COO(CH_3)$　"　"　77.50, "　10.34.
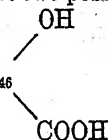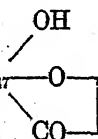
Presence of a Carboxyl Group in Ursolic Acid—That ursolic acid contains a carboxyl group is shown by the facts that it is easily and quickly titrated in absolute alcohol in the presence of phenol-

phthalein with alcoholic sodium or potassium hydroxide, that it yields carbon dioxide by a zinc dust distillation, and that it may be converted into a methyl ester. That the methyl derivative is a true ester may be questioned possibly on the ground that it was formed by the methyl iodide or dimethyl sulfate method and was not prepared through esterification with acid and alcohol. In order to prove, therefore, that an ester was indeeed formed it will be necessary to consider the several possible structures for the parent compound.
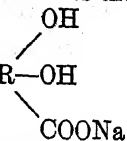
The production of carbon dioxide by zinc dust distillation (7) eliminates a possible phenolic structure, therefore two possibilities remain; namely, an oxy-acid structure $C_{29}H_{46}$ $\begin{matrix} OH \\ \diagup \\ \diagdown \\ COOH \end{matrix}$ and a lactone structure $C_{29}H_{47}$ $\begin{matrix} OH \\ \diagup \\ \diagdown \\ CO \end{matrix}$ —O—. Nooijen (5) and van der Haar (7) have favored the acid structure while Dodge (9) claims the lactone structure. Van der Haar has already pointed out that if it is assumed the structure is lactonic then by the preparation of a salt and from this an ester there must be gained an additional hydroxyl group. He has shown, however, by the Tschugaeff-Zerewitinoff method that the methyl derivative contains only one OH group, which was present before methylation. The presence of only one hydroxyl group in the methylated product is further verified by the formation of a monoacetylmonomethyl compound instead of a diacetylmethyl derivative, which would have to be formed if the lactonic interpretation represented the correct structure.

If we consider the extremely remote possibility that one of the hydroxyl groups in the salt of the alleged lactone, R $\begin{matrix} OH \\ \diagup \\ \diagdown \\ COONa \end{matrix}$ was methylated as in the sugars and from this there was formed a monoacetyl derivative it would still be necessary to prove the

presence of sodium or a free carboxyl group in case the sodium were removed by hydrolysis during recrystallization. This possibility is ruled out, however, by the fact that the mono-acetylmonomethyl derivative which was actually formed failed either to titrate or to show the presence of sodium.

From the reasoning above and a consideration of the nature of the acetylation products which will be the subject of a later communication it will be seen, therefore, that it is not possible to formulate a lactonic structure for ursolic acid. That this substance is a hydroxy acid was further substantiated by preparing from the monoacetyl derivative by means of thionyl chloride an acid chloride which was converted into the corresponding methyl ester by boiling with methyl alcohol. This monoacetylmono-methyl derivative proved to be identical with the derivative prepared by methylating ursolic acid with sodium ethoxide and methyl iodide and following this treatment by acetylation. The formation of a methyl derivative on boiling the product formed by thionyl chloride with methyl alcohol can only take place by assuming that the thionyl chloride reacted with a carboxyl group to form the acid chloride.

Monoacetylmethylursolate, Employing Thionyl Chloride—Mono-acetylursolic acid was treated slowly with an excess of ice-cold thionyl chloride. After several hours, the excess of thionyl chloride was removed by evaporation under reduced pressure and the residue dissolved in ether. The crude acid chloride obtained upon evaporating the ether was then boiled with methyl alcohol. Addition of water to the alcoholic solution caused the separation of the methyl ester. This was reacetylated to insure complete acetylation in case the free hydrochloric acid present should have effected partial hydrolysis. The final product was recrystallized from dilute alcohol. The substance had the same melting point, 246–247°, and yielded the same optical data, as the previously prepared monoacetylmethylursolate. Dr. Edgar T. Wherry and G. L. Keenen kindly made an examination of both preparations, which proved to be identical.

In each case the crystals appeared as platy rods or irregular flakes, without definite angles or cleavages. Refractive indices, $n_\alpha = 1.530$, $n_\beta = 1.537$, $n_\gamma = 1.565$, $n_\gamma - n_\alpha = 0.035$, all ± 0.003. The minimum index was shown lengthwise of rods, and means of

the other two, crosswise in many cases, but grains tilted to show the other two indices were occasionally observed. Between crossed nicols colors of about the second order were obtained, the extinction being parallel and the elongation negative. In convergent light, biaxial figures were frequent, the measured $2E$ being $85° \pm 5°$, and the sign, positive. This agrees well with the value calculated from the indices, $2E = 88° 10'$. Dispersion of the optic axes is distinct, with $2E_{red} > 2E_{violet}$.

Further confirmatory evidence of the identity of the two preparations was obtained by analyzing the compound prepared by using thionyl chloride.

0.0999 gm.: 0.2832 gm. CO_2 and 0.0922 gm. H_2O.
0.1004 " : 0.2837 "　　"　　" 0.0919 "　　"
0.0998 " : 0.2829 "　　"
Found.　C 77.31, 77.06, 77.31; H 10.33, 10.24.
$CH_3CO \cdot O \cdot C_{29}H_{46} \cdot COO(CH_3)$ requires C 77.28, H 10.23.

Phthalylmethylursolate, $(COOH \cdot C_6H_4 \cdot COO) \cdot C_{29}H_{46} \cdot COO(CH_3)$— 1 gm. of air-dried methylursolate was heated 2 hours at 140–160° with an excess of phthalic anhydride. When the mixture was poured into dilute sodium carbonate solution a portion remained insoluble. This was collected, washed with water, and converted into the free acid derivative by solution in alcohol and treatment with dilute hydrochloric acid. The precipitate was collected, washed with water, and dissolved in alcohol, which then was allowed to evaporate spontaneously. A separation of crystalline material occurred, weighing 0.7 gm. when air-dried. The recrystallized substance melted, with previous sintering, at 214–215°. Combustions of the material, dried at 125°, gave the following results.

0.1019 gm.: 0.2827 gm. CO_2 and 0.0807 gm. H_2O.
0.1011 " : 0.2800 "　　"　　" 0.0803 "　　"
0.1027 " : 0.2843 "　　"　　" 0.0787 "　　"
Found.　C 75.66, 75.53, 75.50; H 8.86, 8.89, 8.58.
$(COOH \cdot C_6H_4 \cdot COO) \cdot C_{29}H_{46} \cdot COO(CH_3)$ requires C 75.68, H 8.80.
$(COOH \cdot C_6H_4 \cdot COO) \cdot C_{30}H_{43} \cdot COO(CH_3)$　"　　" 75.90, " 8.93.

Phthalylursolic Acid, $(COOH \cdot C_6H_4 \cdot COO) \cdot C_{29}H_{46} \cdot COOH$—1 gm. of air-dried ursolic acid was mixed with an excess of phthalic anhydride and heated for 2 hours at 140–160°. The mixture

softened and partly melted. It was stirred frequently, cooled, and finally an excess of alcohol was added, and the mixture was further heated on the steam bath. After solution had taken place the mixture was poured into dilute sodium carbonate solution. A trace of the substance remained insoluble. This was filtered off and the filtrate extracted with petroleum ether (b.p. 40–60°). The aqueous portion was acidified with hydrochloric acid and extracted with ether, which process removed phthalyl-ursolic acid. Upon evaporation of the ether, the substance, crystallized from dilute alcohol, weighed 1 gm., air-dried. After recrystallization from dilute alcohol, and drying at 120°, it melted at 264–265°. It was analyzed with the following results.

0.1016 gm.: 0.2803 gm. CO_2 and 0.0777 gm. H_2O.
0.1038 " : 0.2869 " " " 0.0809 " "
Found. C 75.24, 75.38; H 8.56, 8.72.
$(COOH \cdot C_6H_4COO) \cdot C_{29}H_{46} \cdot COOH$ requires C 75.44, H 8.67.
$(COOH \cdot C_6H_4 \cdot COO) \cdot C_{30}H_{48} \cdot COOH$ " " 75.67, " 8.80.

Phenacylursolate, $(C_6H_5COCH_2)CO_2 \cdot C_{29}H_{46} \cdot OH$—Anhydrous ursolic acid (2.3 gm.) was suspended in 70 per cent alcohol and nearly neutralized with sodium hydroxide, after which 1 gm. of bromoacetophenone was added and the mixture boiled $1\frac{1}{2}$ hours. Additional alcohol was added to redissolve the ester which precipitated during the reaction. Cooling under tap water yielded a precipitate, which, dried at 80°, weighed 2.6 gm. A further quantity of 0.25 gm. was obtained from the filtrate upon diluting with water. The substance was recrystallized several times by solution in hot 95 per cent alcohol and the gradual addition of water. Dried at 120°, it melted at 199–200° and gave the following results on analysis.

0.1020 gm.: 0.2964 gm. CO_2 and 0.0863 gm. H_2O.
0.1025 " : 0.2983 " " " 0.0873 " "
Found. C 79.25, 79.37; H 9.47, 9.53.
$(C_6H_5COCH_2) \cdot CO_2 \cdot C_{29}H_{46} \cdot OH$ requires C 79.38, H 9.48.
$(C_6H_5COCH_2) \cdot CO_2 \cdot C_{30}H_{48} \cdot OH$ " " 79.53, " 9.52.

SUMMARY AND CONCLUSIONS

In 1923, a petroleum ether-insoluble compound to which the name malol was given was isolated from apple peels. It possessed the

same formula, $C_{30}H_{48}O_3$, and characteristics as urson from bear-berry leaves and exhibited the same general chemical properties as prunol from the leaves of wild black cherry.

A reinvestigation of urson was reported in 1924 by van der Haar, who suggested the name ursolic acid as being in accord with its chemical character. He claimed for the substance the formula $C_{31}H_{50}O_3$. On the basis of this work, he concluded in separate publications that malol, prunol, and ursolic acid were identical. The formation of the acetylation products was also brought into question.

Because of these discrepancies, it seemed desirable to undertake a more exhaustive chemical study of the three substances. The results of this study are embodied in the present paper and may be summarized as follows:

Malol, prunol, and urson are identical, thus confirming van der Haar's conclusions in this respect. The name ursolic acid has been adopted for the substance.

The formula for ursolic acid appears to be $C_{30}H_{48}O_3$ and not $C_{31}H_{50}O_3$ as claimed by van der Haar. This conclusion is based on the results of 92 combustions including those of the parent substance and many of its derivatives.

The following compounds have been prepared and analyzed: ursolic acid from three sources, the diacetyl derivative of ursolic acid, monoacetylursolic acid, regenerated ursolic acid, methylursolate, monoacetylmethylursolate, regenerated methylursolate, phthalylursolic acid, phthalylmethylursolate, and phenacylursolate. The averaged values of numerous combustions of these substances agree more closely with the C_{30} formula than with the C_{31} formula.

The preparation of monoacetylmethylursolate by treating monoacetylursolic acid with thionyl chloride and boiling this product with methyl alcohol constitutes further confirmatory evidence of the hydroxy acid structure of ursolic acid.

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PHOSPHORUS DISTRIBUTION, SUGAR, AND HEMO-
GLOBIN IN THE BLOOD OF FISH, EELS,
AND TURTLES

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INTRODUCTION

Little information is available concerning the chemical composi-
tion of the blood of the lower vertebrates. This is probably due to
the general difficulty of obtaining unclotted samples. The blood
of all species that have nucleated erythrocytes has the common
property of very rapid clotting after it is removed from the body.
Among these the blood of the turtle is probably the slowest to clot,
while fish and eel blood are at the other extreme.

The phosphorus distribution in the blood of the lower vertebrates
is of special interest since the nuclei are very rich in this element.
Nasse (1) was probably the first to recognize the high content of
phosphorus in the blood of animals whose red cells are nucleated.
He showed the blood of chickens and geese to be very rich in total
phosphorus. In two preliminary reports we have shown that fish
blood is also very rich in total phosphorus (2, 3). These findings
were of special interest to us since we had shown previously that
trout will grow to maturity upon meat diets that are very rich in
phosphorus and poor in calcium (4). In this respect fish seem
unique in their nutritional requirements, at least, when judged by
the laws of a science that is based largely upon experiments with
one species, the rat.

Our objective in the present study has been to determine the
phosphorus distribution in the blood of some of the lower cold
blooded vertebrates. We have incorporated a small amount of
other data, such as those upon the phosphorus distribution in the
blood of the cow, in order to show the contrast in composition of

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two very different vertebrates. At the same time we have included values upon the sugar and hemoglobin of blood of these lower vertebrates in the cases in which they may throw some light upon the variations in phosphorus that we have discovered.

Methods

Phosphorus was determined by the method of Denigé (5) under the conditions elaborated by Kuttner and Cohen (6). We have introduced two slight modifications which have simplified the method considerably. In the first place we have eliminated the frothing that accompanies the digestion of fresh organic material with sulfuric acid by allowing overnight heating in the drying oven at 110°. After such a period of preliminary heating a sample can be digested on the "medium" unit of the ordinary electric hot plate with little attention. In the second place we have employd perchloric acid (60 per cent) in place of hydrogen peroxide (superoxol) as an oxidizing agent. This reagent is easily procured free from phosphates. Only about one-fourth as much is required for a given oxidation and the excess is easily removed by heating. The following procedure is typical of that employed upon fish blood. For the determination of total phosphorus 1 cc. of blood is made up to a volume of 40 cc. with distilled water. This is allowed to stand in a warm laboratory overnight. It is then mixed thoroughly and an aliquot of 1 cc. transferred to a test-tube graduated at 10 cc. A quartz pebble and 0.5 cc. of 10 N sulfuric acid are added and the tube placed in the drying oven overnight. 3 drops of perchloric acid are then added and the tube placed on the hot plate at the "medium" heat until colorless. The excess perchloric acid is removed by heating at the "high" temperature for 20 to 30 minutes. The point of removal of the last trace of perchloric acid is easily detected by the smooth boiling of the sulfuric acid in contrast to the slight bubbling in the presence of a trace of perchloric acid. The remainder of the determination is carried out in the usual manner described by Youngberg (7). We have been able to secure quantitative recoveries of added phosphate by this method which show that none is lost in the heating. If the sample is digested for a period of 2 hours or more at the hottest temperature of the hot plate, which is a dull red, there may be a slight loss of phosphorus.

TABLE I

Phosphorus Distribution (Mg. per 100 Cc.) in Fish and Turtle Blood Compared with Beef Blood

Specimen	Whole blood	Total P, red cells	Cells			Plasma				Hematocrit
			Lipoid	Acid-soluble inorganic	Total acid-soluble	Total	Lipoid	Acid-soluble inorganic	Total acid-soluble	
Pike										
Mean............	87 (9)	147 (3)	27 (3)			44 (5)	21 (5)	18 (5)	23 (5)	27 (5)
Maximum.........	130	160	28			52	28	24	28	33
Minimum.........	52	131	26			28	9	10	16	16
Carp										
Mean............	92 (14)	199 (5)	25 (5)	39 (5)	96 (5)	34 (5)	18 (5)	6.6 (5)	8.9 (5)	32 (5)
Maximum.........	120	232	32	50	114	51	21	10	12.1	44
Minimum.........	57	163	22	28	55	24	16	4	4.4	25
Bullhead*..........	77	238	13	11	95	22	12	7.0	9.0	26
Snapping turtle (Chelydra serpentina)										
Mean............	49 (6)	195 (6)	15 (6)	12 (6)	63 (6)	11 (6)	5 (6)	4.1 (6)	4.4 (6)	22 (6)
Maximum.........	57	242	26	33	82	13	6	5.2	5.7	27
Minimum.........	34	180	11	5	30	8	3	3.3	3.7	11
Beef										
Mean............	22.9	29.0	14.2	3.1	12.5	18.3	11.4	5.6	5.9	41

The number of samples analyzed is shown in parentheses.
* The sample was a pooled one from ten bullheads.

In the fractionation of the blood we have follôwed the current procedure, detailed by Youngberg (7). The acid-soluble fractions were prepared by removing proteins with trichloroacetic acid and the lipid fractions by the use of the alcohol-ether mixture.

Hemoglobin was determined by the method of Wong (8). The reducing values were obtained by the Somogyi modification of the Shaffer-Hartmann method (9).

Blood samples were obtained from fish and eels by cardiac puncture. All samples were taken as soon as possible after removal of the animals from water. No anesthesia was employed with the fish or turtles but chloretone was used for all lamprey-eels. Turtle blood was taken by severing the blood vessels of the neck. Samples were taken from the congo-eels without preliminary anesthesia. Potassium oxalate was used to prevent clotting in most cases. In the later work sodium citrate was favored since it seemed to cause the loss of fewer samples from hemolysis.

The plasma of both fish and turtle blood is slightly yellow like that from the cow. We rejected samples that were hemolyzed since we felt that hemolysis might cause serious errors in the phosphorus distribution. We have used three different fish species of widely different food habits, the pike (*Esox lucius*), the carp (*Cyprinus carpio*), and the common bullhead (*Ameiurus nebulosus*). Snapping turtles (*Chelydra serpentina*) were the ones used in this case. The turtles included both sexes and two of the females were filled with mature eggs.

Results

In Table I we have shown the contrasts in the phosphorus distribution in blood with and without nucleated erythrocytes. The values for beef blood represent the mean for three samples taken from Holstein cows at the peak of the lactation period. The greatest differences are found in the red blood cell constituents. The plasma of pike blood is characterized by high phosphorus values. Whether this is a reflection of the greater activity of the pike under natural conditions compared with the carp or whether it is associated with the rapid death of the former when removed from water we cannot say. Embden (10) has shown that the trout muscle loses its power of synthesizing hexosephosphoric acid much more rapidly than carp muscle. Every fisherman knows

that pike die quickly when taken from the water while carp arrive at the city markets alive after an overnight shipment in barrels with no water. Turtle blood is low in lipid phosphorus. In our previous report (2) we have shown that there is much less cholesterol in turtle blood than in fish blood. This indicates that there is not only a relationship between the phospholipids and cholesterol in the blood of individuals of the same species but that there is also a relationship in blood of characteristic compositions from different

TABLE II

Iron, Hemoglobin, Red Blood Cells, and Sugar in Fish and Turtle Blood

Specimen	Weight of specimen	Fe	"Blood sugar"		No. of red blood cells	Red cells in whole blood
			Whole blood	Plasma		
	kg.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*		*vol. per cent*
Pike						
Mean...........	1.7 (9)	33 (8)	142 (8)		1,097,000	27 (5)
Maximum.......	2.6	40	281		1,372,000	33
Minimum.......	1.1	25	35		404,000	16
Carp						
Mean...........	2.9 (7)	35 (11)	69 (4)	93 (4)	1,022,000	32 (5)
Maximum.......	5.0	51	92	110	1,328,000	44
Minimum.......	1.6	18	53	81	280,000	25
Bullhead (pooled sample).........		29	41			26
Snapping turtle						
Mean...........	1.8	20 (9)	33 (7)	41 (7)	356,000	22 (6)
Maximum.......	2.6	23	56	68	622,000	27
Minimum.......	0.7	17	29	26	218,000	11

species. We were unable to establish any correlations between the sex or sexual cycle of either turtles or fish, and the blood composition.

Since Hall and coworkers have shown (11) that asphyxiation increases the hemoglobin, blood sugar, and iron in fish blood, we have measured these constituents in the same blood samples as those used for the phosphorus determinations, whenever the volume of blood was sufficient. We have done this hoping to

determine whether the phosphorus differences between carp and pike blood are the result of asphyxiation or are real species differences. We have summarized these data in Table II. Although the iron and red blood cells show similar values in carp and pike blood, the blood sugar values are much higher for the latter. This tends to confirm Gray's findings that there is a relation between the activity and the blood sugar level in fish (12). Most of the reducing substances in both fish and turtle blood are concentrated in the plasma. We first found this by calculations based upon the values for the whole blood and plasma. We then confirmed it by direct measurements upon the cells of turtle and carp blood. In both cases the erythrocytes contained only a trace of reducing substances and these traces were probably the contamination from the plasma which we did not wash from the centrifuged cells.

Blood of Lamprey-Eel

In his study of the life history of the lamprey-eel Gage (13) has shown the marked contrasts in the food habits and living conditions during the various periods of its life. The first 4 to 5 years of its life are passed in a mud bank; it feeds upon water organisms during the larval stage. During the adult period it swims into the lake and grows to maturity upon no other food than fish blood. As a mature adult it ceases to eat, migrates upstream for a short distance, spawns while in excellent physical condition, and then undergoes a rapid physical degeneration in the course of a few days and dies.

Since the larvæ are only about the size of earthworms it is very difficult to procure sufficient blood for analysis. From fifteen larvæ we obtained a gm. of blood. This showed a phosphorus content of 30 mg. per 100 cc. This value is very low for blood with nucleated cells.

In order to study the phosphorus distribution in the blood of adult lampreys when they are in prime physical condition just before the spawning period and just at the spawning period we have prepared two pooled samples by taking 1 cc. of blood from each of eight lampreys just before the spawning period and a similar sample 1 week later. In Table III we have summarized these data upon the phosphorus distribution. They show a

tendency for the total phosphorus of the whole blood to decrease but for the cell composition to remain constant.   In a second study we took blood from four individuals in prime condition at the spawning period and from four others that were exhausted after spawning and within a few days of death.   Upon these blood

TABLE III

Phosphorus Distribution (Mg. per 100 Cc.) in the Blood of the Adult Lamprey-Eel

Date	Whole blood	Cells, total P	Plasma		
			Total	Acid-soluble	Inorganic
May 24..........................	51	136	27	14	7
June 3..........................	41	137	21	13	

TABLE IV

Blood Composition of the Lamprey-Eel during the Periods of Spawning and Just before Death

Condition	Hb	"Glucose"	Total P
	gm. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*
Spawning..........................	9.1	123	37
Exhausted..........................	7.0	54	39

TABLE V

Blood Composition of the Congo-Eel

	Congo-eel 1	Congo-eel 2
Erythrocytes per c.mm..........................	25,000	28,000
Hematocrit......................................	40	26
"Glucose," *mg. per 100 cc*......................	32	25
P in whole blood, *mg. per 100 cc*...............	46	37
Plasma P, *mg. per 100 cc*.......................	10	11
" lipid P, *mg. per 100 cc*................	7	5

samples we determined hemoglobin, total phosphorus, and the reducing value.   These results are summarized in Table IV.

Blood of Congo-Eel (Amphiuma tridactylum)

Since the blood samples mentioned above contain nucleated cells of moderate size we wished to compare the composition of some

with very large erythrocytes such as those of *Amphiuma*. Only two specimens were available, however, and the small amount of blood available made an accurate analysis of the cells impossible. In Table V we have shown the values obtained. The phosphorus values are quite similar to those of the lamprey-eel blood.

SUMMARY

The total phosphorus in the blood of animals with nucleated erythrocytes is higher than that in blood with non-nucleated cells. The blood of carp and pike has approximately 4 times as much phosphorus per unit volume as beef blood. The distribution of phosphorus in the blood of fish has been determined. The plasma phosphorus of pike blood is higher than that of carp. The blood sugar is also higher in pike blood. This may be a species characteristic related to activity or may be associated with the differences of the two species in their resistance to asphyxiation.

The phosphorus distribution in turtle blood differs from that of fish blood in the low values for the plasma constituents. The phospholipids of turtle blood are especially low. These are associated with the low cholesterol values that have been shown previously in turtle blood.

The blood of eels is lower in phosphorus than fish blood but higher than that of cattle. The blood changes that accompany the death of the lamprey-eels after spawning show some lowering of the phosphorus values but the changes are chiefly concerned with glucose and hemoglobin decreases. Blood with large nucleated cells contains the same amount of total phosphorus as that with small cells.

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THE DESTRUCTIVE ACTION OF FINELY DIVIDED SOLIDS
ON VITAMIN A*

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New York*)

During some work with a vitamin concentrate of cod liver oil
it was found that comparatively short storage at room tempera-
ture of a mixture of the concentrate with granulated lactose,
as it is used in the manufacture of tablets, resulted in destruction
of vitamin A. This result was unforseen inasmuch as the stability
of vitamin A in aged samples of cod liver oil and other vehicles
had on occasion received experimental confirmation. There was
no basis apparent for assuming a specific destructive action on the
part of lactose.

In 1924, Dunn (1) recorded in a foot-note that when cod liver oil
was mixed with starch, the mixture granulated, and then stored
in corked bottles in the dark for 6 months, vitamin A was destroyed.
Somewhat later Adams, Anderegg, and Nelson (2) observed that
synthetic rations containing added cod liver oil evolve a pene-
trating odor resembling that of acrolein.[1] They speculated on
the possibility of the failure of these rations to protect against
xerophthalmia being explicable on the basis of the destruction of
vitamin A. These references when considered in conjunction
with our experience with granulated lactose pointed to the possi-

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* Presented before the Medicinal Section of the 79th meeting of the
American Chemical Society at Atlanta, Georgia, April 9, 1930.

[1] Anderegg and Nelson (3) noted a characteristic odor of decomposition
when cod liver oil was added to dry powders such as skimmed milk, whole
milk, starch, or dextrin. Addition of 5 per cent ether, chloroform, or
benzene did not prevent the decomposition, while 95 per cent of alcohol,
wheat germ oil, or water had a protective action in the order given. Pro-
tection with water varied with the amount added. With 10 per cent water
no decomposition was evident even after several months.

bility of the destruction of vitamin A being a reaction which is general for mixtures of vitamin A with all finely divided solids. The purpose of this investigation was to throw some light on this matter.

A vitamin concentrate of cod liver oil, prepared by our process(4) was used throughout as a source of vitamin A. Fourteen powdered substances of different chemical constitution were used for the mixtures with vitamin A. The following procedure was used in the investigation. 0.5 cc. of an ether solution of the unsaponifiable fraction from 15 gm. of cod liver oil was added to 5 gm. of the powdered substance in a 2 ounce narrow mouthed amber bottle. The ether was distilled off with a water vacuum pump, with the bottle immersed in a water bath at about 35°. The vacuum was broken with carbon dioxide or air, and the bottle was stoppered and stored.[2] Thus, the vitamin A equivalent of 15 gm. of cod liver oil as contained in 0.11 gm. of unsaponifiable matter was distributed over 5 gm. of powder. The vitamin A value of the mixture was determined by extracting the powdered substance with a definite amount of chloroform and submitting the chloroform extract to the well known antimony trichloride color test in a Lovibond tintometer. The color value was determined for each mixture, first when freshly prepared and after suitable periods of storage. The decrease in intensity of blue color was taken as a measure of the destruction of vitamin A. The numerous agreements recorded in the literature between the antimony trichloride color test and the results of the biological assay make it fairly certain that the conclusions reported in this paper are a true representation of the fate of vitamin A. Indeed, in a few instances where we performed the biological assay the results were in general agreement with the predictions of the color test.

Table I shows the destruction with the powders used over various periods of storage. The results are given for storage under carbon dioxide, and in some cases both for carbon dioxide and for air. It may be concluded from Table I that:

1. Destruction of vitamin A is general with all the powders used, though the rate of destruction varies.

2. The degree of destruction is the same regardless of whether the mixture is stored under carbon dioxide or air.

[2] All the bottles were stored in a dark closet.

3. The destruction of vitamin A is progressive, as indicated in the results with hydroquinone and sodium hypophosphite.

In view of the lability of vitamin A to oxidation, it seemed plausible to ascribe its destruction to the air adsorbed on the large surface of the powders over which the vitamin was distributed. To test this assumption we ran an experiment with nuchar, a finely divided vegetable char which presumably contains an excessive amount of air adsorbed on its vast surface. We found that over 90 per cent of the vitamin was destroyed within 3 hours after mixing

TABLE I

Effect of Storage on Mixtures of Vitamin A with Powdered Materials

Powder used	Time of storage	Destruction of vitamin A	Gas under which mixture was stored
	days	*per cent*	
u. s. p. basal diet for vitamin A assay....	10	85	Air or CO_2
Lactose granulation...................	8	90	" " "
Iron powder (by hydrogen)............	15	100	CO_2
Ferrous sulfate (dried powder).........	15	100	"
Ferric oxide (ppt. brown powder)......	15	100	"
Calcium hypophosphite................	15	100	"
Magnesium oxide (heavy).............	15	35	"
" citrate....................	15	50	"
Hydroquinone........................	15	15	"
"	47	70	Air or CO_2
Calcium carbonate (ppt.).............	23	90	" " "
Sodium bisulfite......................	19	100	" " "
" hypophosphite................	17	40	CO_2
" "	49	80	Air or CO_2
" pyrophosphate................	26	80	CO_2

the concentrate with the nuchar. This unprecedented rate of destruction seemed to substantiate the assumption of destruction by oxidation. It should be noted that during the process of distilling off the ether from the powders, *in vacuo*, it is probable that only a small fraction of air adsorbed on the powders was removed. It is well known that very high temperature, unusually low vacuum, and long treatment are necessary to effect anything approaching quantitative removal of adsorbed gases from a solid. In our experiments none of these conditions prevailed. Thus, any air originally present in the powders was most likely still available after the vacuum distillation for possible interaction with vitamin A.

To put the oxygen destruction theory to further test, we deemed it necessary to remove the oxygen from nuchar, and then determine whether the anaerobic char would protect the vitamin. A quantity of nuchar was placed in a hard glass combustion tube, open at both ends, through which was passed a steady stream of hydrogen. The tube was heated to redness for 4 hours during the passage of the hydrogen and then cooled to room temperature under the same gas. The vitamin concentrate was added to the reduced powder under anaerobic conditions. The color test applied 3 hours after mixing showed that 90 per cent of vitamin A had been destroyed. The reduced char was as destructive to vitamin A as was the char before treatment with hydrogen. In interpreting this experiment we must bear in mind that vitamin A may contain reactive groups such as −OH or −CHO or unsaturated linkages. It is possible, then, that the vitamin A was destroyed by reaction with hydrogen under the catalytic influence of the powdered char. In other words, nuchar may act as a catalyst both in the oxidation and in the reduction of vitamin A. In order to throw more light on this matter it would be necessary to replace the gases in char with an inert gas such as nitrogen before mixing with the vitamin A concentrate. It is, of course, possible that vitamin A destruction would still take place even in the entire absence of oxygen or other gases. It may be that powdered char and other finely divided substances bring about a condensation or polymerization of vitamin A by virtue of the catalytic influence of surface effects. Experiments are now under way to extend our knowledge in this direction.

The amount of vitamin A we are dealing with in these experiments is very small. The unsaponifiable matter from 15 gm. of cod liver oil amounts to 0.1 gm. The amount of vitamin A present in the unsaponifiable fraction of a potent cod liver oil is probably 1 per cent at the most (5). Thus in 0.1 gm. of unsaponifiable matter we had 1 mg. or less of vitamin A which was distributed over 5 gm. of powder in the case of the thirteen substances listed in Table I and over 2 gm. of nuchar (only 2 gm. of nuchar were used because of its comparatively greater bulk). It has been estimated that the particles in 1 gm. of char possess an area of 120 to 1200 square yards. In 2 gm. of nuchar, then, we had 1 mg. or less of vitamin A distributed over the vast area of 240 to 2400 square yards. Such an

enormous dispersion of a substance is not inconsistent with a marked enhancement of its reactivity.

Some of the results cited in Table I show that vitamin A undergoes no more rapid destruction when the mixture is stored in an atmosphere of air than when it is kept under carbon dioxide. This supports the assumption that the factors involved in the vitamin destruction are all present in the powder itself. However, it must be noted that in the experiments where the powders were kept under carbon dioxide, the carbon dioxide had a content of about 0.6 per cent of oxygen, the presence of this amount of oxygen being due to the limitations of the water vacuum pump used. Thus, in the 2 ounce bottle used in our experiments the carbon dioxide gas had a content of about 0.5 mg. of oxygen. This

TABLE II

Destruction of Vitamin A in a Lactose Granulation as Modified by Added Substances

Per cent of substance added to lactose granulation	Time of storage	Destruction of vitamin A	Gas under which mixture was stored
	days	*per cent*	
None..............................	8	90	CO_2
Hydroquinone, 1 per cent..........	15	5	"
" 1 " "	45	35	"
Water, 10 per cent................	8	20	"
" 10 " "	63	100	"

0.5 mg. of oxygen was theoretically available for interaction with 1 mg. or less of vitamin A. However, it seems more likely that if the destruction of vitamin A resulted from reaction with the oxygen in the gas outside the powder, the destruction would have been more rapid in the experiments with air where the oxygen concentration was about 35 times as great as that in the bottles containing carbon dioxide.

In Table II are reported the results of some experiments with granulated lactose containing supplements of compounds which we considered might modify the destructive action of the lactose powder on the vitamin.

In the hydroquinone experiment, an ether solution containing the vitamin concentrate together with the hydroquinone was added

to the lactose, and the ether was distilled off as described above. The hydroquinone exerted a marked influence on the vitamin destruction. Only 5 per cent of the vitamin was lost in 15 days compared with 90 per cent lost in half that time when hydroquinone was absent. However, in 45 days the loss of vitamin rose to 40 per cent, indicating that the inhibiting action of hydroquinone is a temporary effect. From these results it might be concluded that the destruction of vitamin A by the granulated lactose is an autoxidation in which the hydroquinone plays the familiar rôle of prolonging the induction period.

In the experiment with water, the lactose granulation was mixed with 10 per cent water, the ether solution of the concentrate was added, and the ether removed in the usual manner. Here, also, as in the experiment with hydroquinone, there was marked inhibition of vitamin destruction. Only 20 per cent of the vitamin was lost in 8 days compared with 90 per cent lost in the same time with the dry granulation. In 63 days, however, destruction was complete. Water, then, is not as effective as hydroquinone in reducing the loss of vitamin.

It is evident that the inhibiting action of water cannot be explained on the same basis as the hydroquinone effect. It is likely that when water is added to the granulated lactose, the granules in absorbing the liquid thereby effect an alteration in the surface of the powder which is reflected in a diminution of its catalytic activity. Our result with water is in harmony with the experience of Anderegg and Nelson (3) who found that the addition of water to dry powders inhibited the decomposition of cod liver oil in admixture with these powders.

Our results raise grave doubt of the validity of many conclusions in the older literature on vitamin A assays, in which it was general practice to incorporate the vitamin A test product with the dry powdered basal ration for a supply of 1 or more months test feeding. Unfortunately, this practice is still current in some quarters. An official method for vitamin A assay should provide for a definite procedure of feeding the vitamin A test product other than by dilution with the powdered basal ration.

An interesting application of our results may be possible in the preparation of a vitamin A-free casein powder by simply storing the dry casein powder for a period of time instead of the present

tedious and expensive method of alcoholic extraction of the casein. Our experiment with the basal ration used in the u. s. p. assay for vitamin A showed 85 per cent destruction of vitamin A in 10 days (Table I).

SUMMARY

Mixtures of a vitamin A concentrate of cod liver oil with finely divided solids undergo destruction of vitamin A on standing. An attempt has been made to elucidate the nature of the destruction.

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PHYSICOCHEMICAL PROPERTIES OF CROCODILE
BLOOD (CROCODILUS ACUTUS, CUVIER)

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The physicochemical properties of blood in those organisms
which have evolved a mechanism for transport of carbon dioxide
and oxygen at constant temperature are well understood. It is an
interesting question as to the degree of similarity between a
system thus developed and one in an organism which has remained
poikilothermous. Such studies as those of Redfield, Coolidge, and
Hurd (1) and of Kerridge (2) on hemocyanin-containing blood,
of Krogh and Leitch (3) on the affinity for oxygen of blood from
various species of fish, and of Austin, Sunderman, and Camack (4)
on the relation of body temperature to serum electrolytes in the
alligator have revealed first order variations in different species.

There are so many variables in blood that when a comparison is
made of two specimens it is often difficult to assign the differences
to particular components of the system. We have attempted to
compare crocodile and human blood and while the comparison is
incomplete it indicates that blood of the crocodile differs greatly
from that of man in several respects and that these differences are
due in part to specific differences in hemoglobin.

Methods—The technique of equilibration and analysis of blood
was slightly modified on account of the lower equilibration tem-
perature (29° in most cases) and the high rate of metabolism in
red blood cells. Equilibration was carried out for 30 minutes in-
stead of the usual 20 minutes; the longer period seemed to be
necessary to secure complete equilibrium between the two phases,
particularly in respect to oxygen. In order to avoid post-equi-
libration changes the two phases were analyzed immediately.
This made necessary the equilibration of one sample of blood at a

time and hence the maintenance of the unequilibrated portion in an unchanged state for many hours. This was accomplished by storing it in a tightly stoppered vessel in crushed ice in an Icy-hot jug. One specimen of blood was held in such a container below 1° for 60 hours without change in oxygen capacity or in carbon dioxide capacity.

The calculation of results was modified by the lower equilibration temperature. The solubility of carbon dioxide in serum and cells at 38° has been established by Van Slyke, Sendroy, Hastings, and Neill (5). We have assumed that the temperature effect on its solubility in blood is the same as in water. The ionic strength of crocodile serum is about the same as that of human serum and one can probably safely assume the same pK'_s value with the usual allowance for temperature effect. A similar assumption for cells is less certain since the concentration and properties of hemoglobin are important in determining the value for pK'_c. In the absence of information regarding either the distribution of hydrogen ions between cells and serum or the value of pK'_c we have used values for $\log \dfrac{(BHCO_3)_b}{(H_2CO_3)_b}$ in place of pH_c.

Blood was obtained from several crocodiles and caimans (*Caiman fuscus*, a related species of reptile). Crocodile A was about 12 feet long. Crocodiles B and C and Caiman A were about 3 feet long. The small specimens had been caught alive and could be handled easily. They were tied to a table, an incision was made under local anesthesia, and blood was drawn from the aorta. Crocodile A was caught on a hook during the night. It was drawn ashore some hours later, shot in the head, rolled onto its back, and blood was obtained by severing an artery in the cardiac area. This blood was nearly saturated with oxygen; evidently it was obtained before serious breakdown of the respiratory and circulatory mechanisms.

These four principle experiments represent three stages of lactic acid accumulation. In Caiman A and in Crocodile C the lactic acid concentration was within the limits observed in man in exercise; in Crocodile A it was 22 milli-equivalents per liter, about twice as high as we have ever found in man. Crocodile B had been injured in capture and was comatose when blood was obtained. Blood leaving its feebly beating heart contained 47 milli-equivalents of lactic acid per liter and almost no oxygen.

Control Experiments on Human Blood—Our most detailed experiment on crocodile blood was on Crocodile A at 29°. Experiments have been carried out on two specimens of human blood at the same temperature and with about the same lactic acid concentration.[1] About one-half the cells were removed from one specimen, thus reducing the cell volume and oxygen capacity to the level characteristic of the crocodile.[2]

Buffer Value of Serum—Some blood from Crocodile A was allowed to clot and the separated serum was equilibrated with carbon dioxide with the results shown in Table I. The buffer value of serum per unit of protein can be calculated by the relation

$$\text{Buffer value} = \frac{-\Delta \text{BHCO}_3}{(\Delta \text{pH}_s)\,(\text{protein})}$$

TABLE I

Separated Serum of Crocodile A at 29°

Calculated buffer value per unit of protein = 0.132. Protein content = 36.9 gm. per liter. $(H_2CO_3) = 0.0385\ pCO_2$.

pCO_2	Total CO_2	Log $\frac{(\text{BHCO}_3)}{(\text{H}_2\text{CO}_3)}$
mm. Hg	*m.-eq. per l.*	
7.0	7.42	1.423
16.7	9.12	1.121
43.4	11.86	0.785
102.3	15.64	0.471

The four sets of values derived from data in Table I fall near to a straight line which corresponds to a buffer value of 0.132. This value is about one-third higher than that of human serum protein at 37.5° in this range of hydrogen ion concentration and, since the determined points cover a wide range, the difference probably is

[1] In order to avoid hemolysis blood is centrifuged and the calculated amount of 85 per cent lactic acid is added to the plasma. This can then be mixed with the cells.

[2] The oxygen-combining capacity of blood from each of three caimans ranged from 3.7 to 4.0 milli-equivalents per liter. In three crocodiles the range was from 3.7 to 4.5. Determinations of red blood cell count, per cent cell volume, and oxygen-combining capacity indicated that the red blood cell of the crocodile has about 3 times the volume of that of man and contains about 95 per cent as much hemoglobin per unit volume.

TABLE II

Whole Blood of Crocodile A at 29°

HbO_2 capacity = 4.13 milli-equivalents per liter.   Lactic acid = 22 milli-equivalents per liter. $(H_2CO_3)_b = 0.037\ pCO_2$.

pCO_2	HbO_2	$(Total\ CO_2)_b$	Log $\dfrac{(BHCO_3)_b}{(H_2CO_3)_b}$
mm. Hg	*per cent*	*m.-eq. per l.*	
6.9	91	4.39	
	(100)	(4.19)	1.185
9.7	77	5.65	
	(100)	(4.72)	1.084
39.4	89	11.82	
	(100)	(11.58)	0.838
41.1	78	12.25	
	(100)	(11.75)	0.828
97.0	100	18.04	0.603
103.0	100	18.79	0.593
6.1	7	5.75	
	(0)	(5.93)	1.405
8.0	20	6.57	
	(0)	(7.04)	1.357
38.7	6	13.86	
	(0)	(13.98)	0.943
40.5	17	13.67	
	(0)	(14.05)	0.924
41.1	7	13.66	
	(0)	(13.83)	0.908
90.4	7	19.46	
	(0)	(19.60)	0.685
97.6	15	19.46	
	(0)	(19.70)	0.647

The values for total CO_2 in parentheses correspond to complete oxygenation or reduction.

significant. At the same time it will be noted that the protein concentration is little more than half that of human serum so that
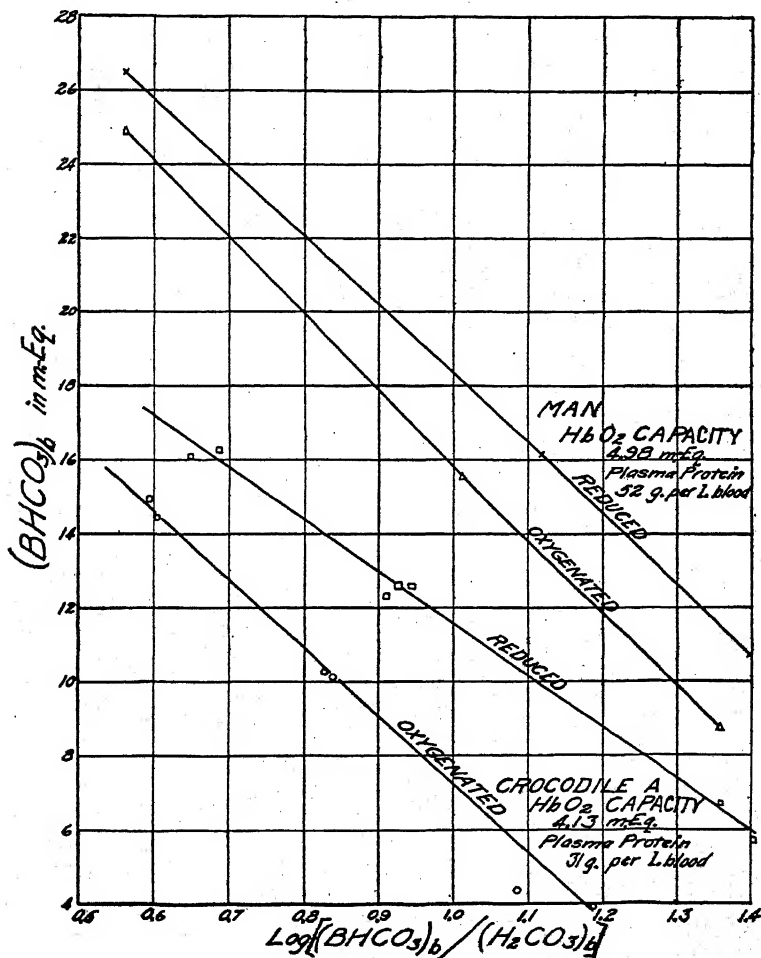
FIG. 1. Bicarbonate of the blood as a function of log $\dfrac{(BHCO_3)_b}{(H_2CO_3)_b}$ in Crocodile A and in human blood at the same temperature and with approximately the same content of hemoglobin and of lactic acid.

the buffer value *per unit of serum* is about three-fourths that of human serum. A similar value for serum protein concentration

was found in Crocodile B, and Austin, Sunderman, and Camack (4) have found low values for its concentration in alligator serum.

Carbonic Acid Capacity of Oxygenated and Reduced Blood—The essential results in an experiment on Crocodile A are given in Table II. From these observations and a similar experiment on human blood, Fig. 1 has been prepared. It is obvious that the alkaline reserve[3] in the blood of Crocodile A has been greatly reduced by lactic acid accumulation but less complete observations on other specimens of blood make possible an estimate of alkaline
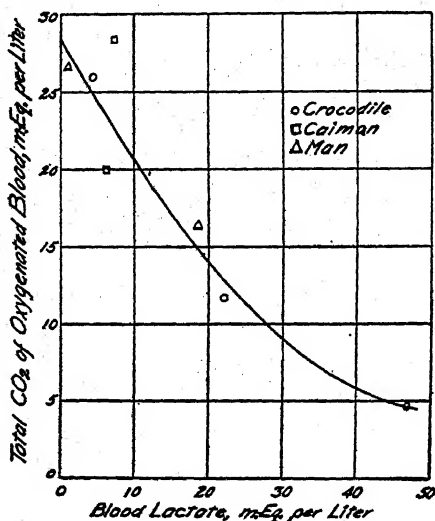
FIG. 2. Variation in carbonic acid capacity of oxygenated blood with lactic acid content. Temperature, 29°; hemoglobin concentration, about 4 milli-equivalents per liter.

reserve in the resting state. Fig. 2 illustrates carbonic acid capacity at a fixed pCO_2 of 40 mm. as a function of lactic acid concentration. It is evident that alkaline reserve of the crocodile at rest is not much different than that of human blood of the same proportion of hemoglobin and at the same temperature.

Fig. 1 shows that the difference in carbonic acid capacity between oxygenated and reduced blood is much greater in blood from Croco-

[3] Arbitrarily defined as the carbonic acid-combining capacity of oxygenated blood at $pCO_2 = 40$ mm.

dile A than in human blood. In order to make a more precise comparison of this difference Fig. 3 has been prepared to show the effect of oxygenation on base bound by protein as a function of log $\frac{(BHCO_3)_b}{(H_2CO_3)_b}$ in various bloods. The results indicate about twice as

great a value for $- \frac{\Delta (BHCO_3)_b}{(HbO_2)_b}$ at constant hydrogen ion con-
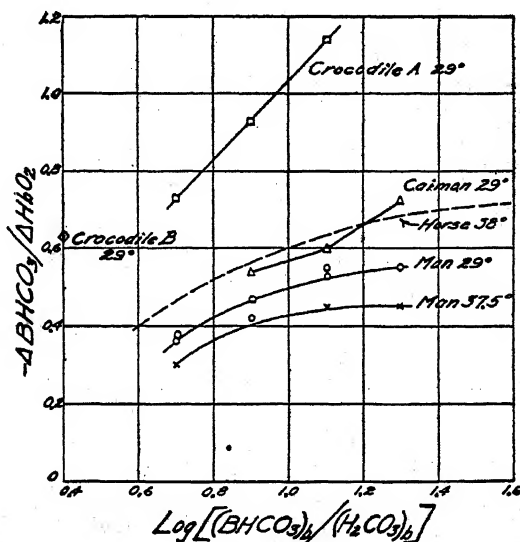
FIG. 3. Effect of oxygenation on the base-binding capacity of hemoglobin as a function of log $\frac{(BHCO_3)_b}{(H_2CO_3)_b}$. The ratio $- \frac{\Delta (BHCO_3)}{\Delta (HbO_2)}$ indicates the increase in equivalents of alkali bound by protein per molecule of oxygen added to reduced hemoglobin. The data on the horse is from Fig. 9, Van Slyke, Wu, and McLean (6).

centration in the crocodile as in man. The value for caiman blood, however, was only slightly greater than that for human blood. It appears quite probable that crocodile hemoglobin differs very considerably from mammalian hemoglobin in this respect.

Such a difference suggests that there may also be differences in buffer value and in affinity for oxygen and the first of these questions will now be considered.

Buffer Value of Oxygenated and of Reduced Cells—Fig. 1 and Table I contain the information necessary for making an approxi-

mate estimate in the case of Crocodile A of buffer value of oxygenated and of reduced cells per unit of hemoglobin. This can be carried out as follows:

$$\beta = \frac{- \Delta \, (BHCO_3)_b - a(P)_s \, \Delta \log \frac{(BHCO_3)_b}{(H_2CO_3)_b}}{\Delta \log \frac{(BHCO_3)_b}{(H_2CO_3)_b} \, (total \, Hb)_b}$$

All concentrations are in milli-equivalents per liter of blood except $(P)_s$ which indicates gm. of serum protein per liter of blood. β represents buffer value of cells per unit of hemoglobin. The value

TABLE III

Buffer Value of Oxygenated and of Reduced Hemoglobin within the Limiting Values for Log $\frac{(BHCO_3)_b}{(H_2CO_3)_b}$ of 0.6 and 1.1

	β_O	β_R	$\dfrac{\beta_O}{\beta_R}$
Human blood (F. A.) at 29°.............	3.09	2.69	1.15
" " (W. C.) " 29°.............	3.01	2.61	1.15
" " (A. V. B.) " 37.5°.....	2.86	2.48	1.15
Crocodile A blood, at 29°..................	3.47	2.40	1.44
Caiman blood " 29°..................	3.47	2.76	1.26

$\dfrac{\beta_O, \, crocodile}{\beta_O, \, human}$, at 29°, 1.14.

$\dfrac{\beta_R, \, crocodile}{\beta_R, \, human}$, at 29°, 0.91.

of 0.132 was used for the constant a in the case of the crocodile and other values similarly derived were used for man and for the caiman. The results of such calculations are given in Table III. While the buffer values are all of the same order of magnitude, it appears that oxyhemoglobin of the crocodile has one-seventh greater buffering capacity than that of man while reduced hemoglobin has about one-tenth less buffer value than that of man.[4]

[4] Crocodile cells are nucleated and in comparison with human cells contain less hemoglobin and more solids. Hence they contain more non-hemoglobin proteins than human cells and the buffer values as calculated are modified on this account. The difference between oxygenated and reduced blood, however, can be ascribed only to hemoglobin.

Human oxyhemoglobin is one-seventh greater in buffer value than reduced hemoglobin while in the crocodile it is nearly one-half greater. However, the experimental observations are not sufficiently numerous to define the curves in Fig. 1 precisely and one can be certain only of the general qualitative conclusions (a) there

TABLE IV

Composition of Plasma and Cells in Oxygenated Blood of Crocodile A When

$$Log \frac{(BHCO_3)_b}{(H_2CO_3)_b} = 0.80$$

	Cells	Plasma
H₂O, *cc. per l*	726	959
Na, *m.-eq. per l*	39.4	149.0
K, " " "	91.2	7.9
Ca, " " "		6.8
Mg, " " "		3.7
Σ cations, *m.-eq. per l*	130.6	167.4
Cl, *m.-eq. per l*	80.5	117.0
HCO₃, " " "	9.7	11.2
$HPO_4^= + H_2PO_4^-$, *m.-eq. per l*	3.9	5.6
Lactate, " " "	16.2	23.5
Proteinate, " " "	20.3	10.1
Hb, " " "	19.3	
Protein, " " "		39.3

Calcium and magnesium were determined on the ash of about 80 cc. of serum according to methods for plant ash of the Association of Official Agricultural Chemists (Methods of analysis, Washington (1925)). Sodium chloride and potassium chloride were obtained from the ash of 10 to 20 gm. of serum and of cells by familiar procedures and potassium was then determined gravimetrically as the chloroplatinate. Phosphate was determined in serum and lactic acid in whole blood; the concentrations of phosphate in cells and of lactate in cells and in serum were calculated on the assumption that the distribution of these ions is the same as that of the chloride ion.

is a greater difference in buffer value between oxyhemoglobin and reduced hemoglobin in the crocodile than in man, (b) serum proteins of the crocodile have a somewhat greater buffer value than those of man while the order of magnitude of buffer value of cell proteins is the same, and (c) the buffer values of *serum* and of *whole blood* are much lower than in man because of low concentration of protein in serum and of cells in blood.

Composition of Plasma and Cells—The concentration of cations and anions in plasma and cells of Crocodile A (Table IV), aside from the high lactate and low bicarbonate values, is not outside the limits observed in mammalian blood. As compared with

TABLE V

Anion Distribution at 29°

Subject	$Log \dfrac{(BHCO_3)_b}{(H_2CO_3)_b}$	HbO_2	$^rCl^-$	$^rHCO_3$
		per cent		
Man (F. A.). HbO₂	1.356	100	0.64	0.83
capacity, 4.98 m.-	1.010	100	0.80	0.97
eq. per l.; lactic	0.562	100	0.96	1.45
acid, 18 m.-eq. per l.	1.398	25	0.72	0.91
	0.561	11	0.99	1.30
Man (W. C.). HbO₂	1.210	100	0.71·	0.84
capacity, 8.2 m.-eq.	0.973	100	0.80	1.01
per l.; lactic acid,	0.573	100	0.93	1.20
19 m.-eq. per l.	1.347	19	0.75	1.01
	1.048	12	0.85	1.07
	0.602	10	0.96	1.30
Crocodile A. HbO₂	1.134	77	0.79	1.20
capacity, 4.13 m.-eq.	0.848	78	0.91	1.20
per l.; lactic acid,	0.593	100	0.99	1.20
22 m.-eq. per l.	1.325	20	0.84	2.10
	0.937	6	1.00	2.10
Crocodile B. HbO₂	0.461	12		4.80
capacity, 3.73 m.-	0.350	90		1.70
eq. per l.; lactic				
acid, 47 m.-eq. per l.				
Caiman A. HbO₂	1.039	100		1.00
capacity, 3.90 m.-eq.	1.089	4		1.40
per l.; lactic acid,				
6.2 m.-eq. per l.				

human cells, potassium is lower and sodium higher. The cell membrane probably is impermeable to these ions because of the large concentration gradients between cells and serum. The sum of cations in cells and in plasma is about one-tenth higher than in man and there is a corresponding difference in chloride concentra-

tion. The estimates of base bound to serum and cell colloids have been made by difference and hence are inexact since they sum up all errors in determination of other ions.   The amount of base bound by hemoglobin is small since an allowance must be made for base bound to non-hemoglobin proteins and to non-diffusible phosphorus compounds.   On the other hand the reac-
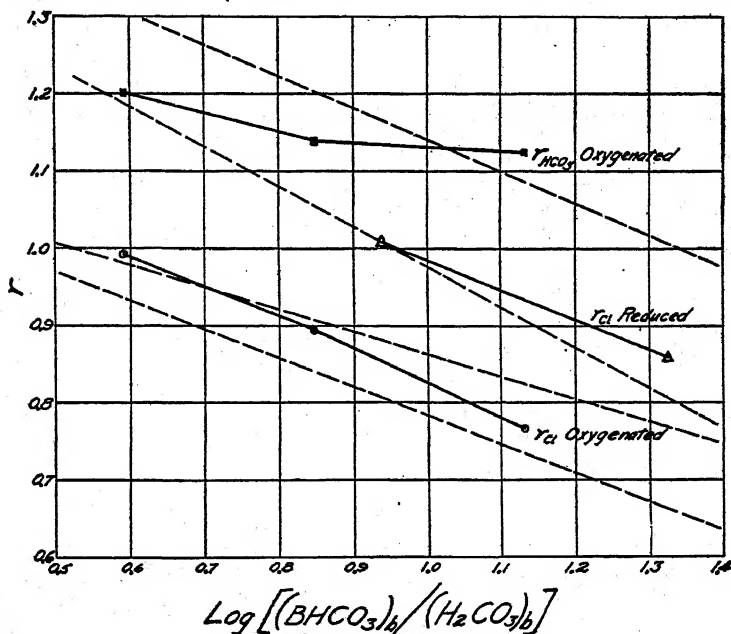
FIG. 4. r_{HCO_3} and r_{Cl} in relation to HbO_2 and log $\dfrac{(BHCO_3)_b}{(H_2CO_3)_b}$, at 29°.
The broken lines are based upon two experiments on human blood at the same temperature.

tion is not far from the isoelectric point of mammalian oxy-hemoglobin.

Distribution of Anions between Cells and Plasma—It has been shown by Van Slyke, Wu, and McLean ((6) Equation 14) that the distribution of anions between cells and plasma can be expressed approximately in terms of base of cells and plasma, base combined to protein in cells and plasma, and hemoglobin of cells. If this equation is applied to crocodile blood, the values given in

Table IV being used, it yields a value for r of 0.88 which is about the same as that derived by Van Slyke, Wu, and McLean ((6) Fig. 1) for horse blood. It will be interesting to see how closely the experimental values agree with this calculation. The results on two specimens of human blood at 29° and on crocodile and caiman blood are given in Table V. The values for human blood have been plotted as a function of $\log \frac{(BHCO_3)_b}{(H_2CO_3)_b}$ and straight lines drawn to correspond to values for r_{Cl} and r_{HCO_3} in completely oxygenated and in completely reduced blood. Such lines have been reproduced as broken lines in Fig. 4 and the experimental values for these ratios in crocodile blood (after correction to complete oxygenation or complete reduction) are plotted on the same figure. The values for chloride ratio are more precise than bicarbonate ratio because (a) direct determination can be made in cells, (b) the total concentration is several times as great as bicarbonate concentration, and (c) one has a check on the accuracy in each pair of determinations since $(Cl)_c + (Cl)_s = (Cl)_b = $ a constant for a given specimen of blood. The values for $(Cl)_c$ and $(Cl)_s$ met the last requirement and it is probable that the calculated ratios are nearly correct. By comparison with values for r_{Cl} in oxygenated blood with corresponding values for r_{HCO_3}, it appears probable that these also are nearly right. There is doubt, however, regarding the accuracy of the high values obtained for r_{HCO_3} in reduced blood (Table V) notwithstanding the fact that all the experimental evidence on this question is in agreement. Thus similarly high values were obtained on two other specimens of blood. In every determination of total CO_2 content of true plasma, a lower value was found than in the corresponding whole blood. If these observations are correct, they suggest a specific combination of carbon dioxide with hemoglobin in reduced blood. The isoelectric point may be at a much more alkaline reaction than in oxyhemoglobin. The values for r_{Cl} and r_{HCO_3} in oxygenated blood are somewhat higher than in human blood and much higher than experimental values for horse blood found by Van Slyke, Hastings, Murray, and Sendroy (7). Since our calculations showed about the same theoretical value for r in crocodile blood as in horse blood, one of the assumptions must have been wrong. The only important assumption which does not rest on reliable experimental evidence

is the concentration of base bound to cell proteins. It is probable, in view of these considerations, that the value for (BP)$_c$ obtained by difference in Table IV is much too high.

Affinity of Hemoglobin for Oxygen—It has been pointed out by Henderson (8) that there is a necessary relation between the facts
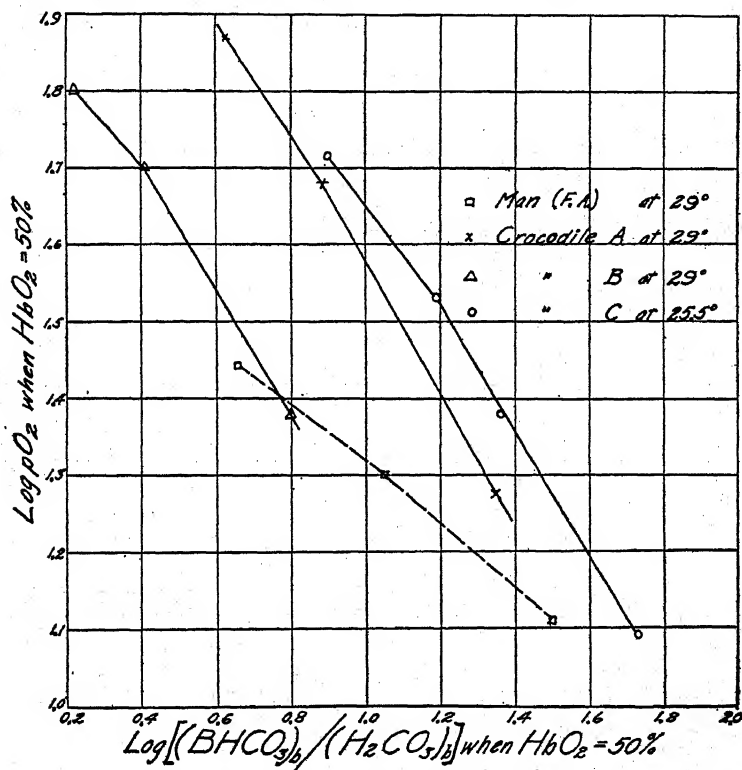
FIG. 5. Position of the oxygen dissociation curves as a function of log $\dfrac{(BHCO_3)_b}{(H_2CO_3)_b}$.

(*a*) oxyhemoglobin is a stronger acid than hemoglobin, and (*b*) the affinity of hemoglobin for oxygen depends upon the hydrogen ion concentration. It has been found by Dill, Edwards, Florkin, and Campbell (9) that there is the same difference in carbon dioxide-combining capacity between oxy- and reduced hemoglobin in man and the dog and also the same effect of hydrogen ion concen-

tration upon the position of the oxygen dissociation curve.   In unpublished observations of the blood of an elasmobranch (*Raia oscillata*) both effects have been absent or nearly so.   Since the change in acid strength when hemoglobin is oxygenated is about
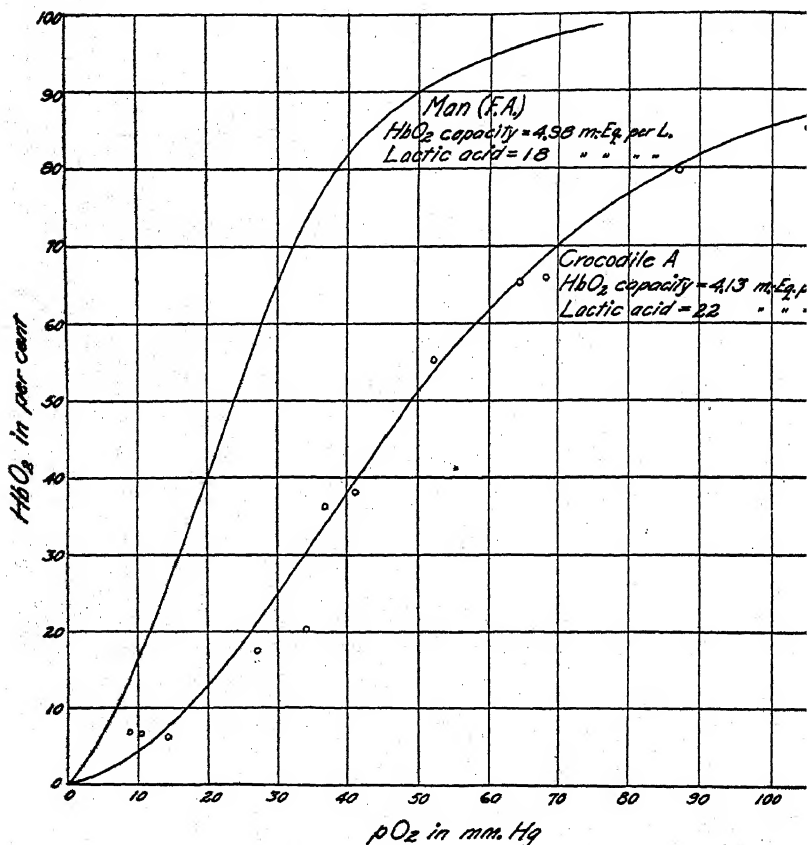
FIG. 6. Form of the oxygen dissociation curves of human blood and of crocodile blood at the same temperature and reaction.

twice as great in crocodile as in human blood, it follows that there should also be a greater effect of reaction change upon affinity of hemoglobin for oxygen. This is shown to be the case in Fig. 5, based upon the examination of one specimen of human blood and

three specimens of crocodile blood. The latter have a uniform change in affinity with reaction. The slope a of the line

$$\text{Log } pO_2 = a \log \frac{(\text{BHCO}_3)_b}{(\text{H}_2\text{CO}_3)_b} + c$$

is nearly twice as great as in man. This conclusion, based upon fairly complete studies of three specimens of crocodile blood, is quite in harmony with the observation that there is an unusually large effect of oxygenation on acid strength of hemoglobin.

The form of the oxygen dissociation curve has been compared with that of human blood at the same reaction and temperature in Fig. 6. If comparison is made of normal crocodile blood at 29° with normal human blood at 37.5° it will be found that the position and form are, as a first approximation, the same.

Physiological Observation—In two cases simultaneous samples of lung air and of arterial blood were obtained. These indicated that the pressure-head of carbon dioxide from blood to lungs is no more than 2 or 3 mm. An oxygen pressure-head from lungs to blood of 20 to 30 mm. was found, even when the saturation of arterial blood was low. Dill and Edwards (10) have reported the rate of uptake of oxygen from the lungs in a small crocodile. The ability of the crocodile to hold its breath, as shown by Parker (11), probably signifies an inertness of the respiratory center to changes originating from carbon dioxide accumulation. On account of this physiological difference and the great effect of acid on lessening the affinity of hemoglobin for oxygen, the crocodile is well equipped to utilize all the available oxygen in the lungs.

SUMMARY

Blood of the crocodile differs from that of man in several respects. Concentration of protein in serum and of hemoglobin in blood is lower. The buffer value of serum protein is somewhat higher and of hemoglobin about the same, the net result being that the buffer value of blood is much less. The most conspicuous difference is in the acid effect on hemoglobin. This is much greater in the crocodile and, correspondingly, oxygenation has a greater effect on the acid-combining capacity of hemoglobin.

We are indebted to Professor Thomas Barbour and to Mr. James Zetek, Custodian, for the opportunity of working at the Barro Colorado Laboratory. Mr. William Consolazio of the Fatigue Laboratory rendered technical assistance and Mr. Donato Carillo helped us in securing material.

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HEMOGLOBIN PRODUCTION

II. THE RELIEF OF ANEMIA, DUE TO MILK DIET, BY FEEDING AMINO ACIDS*

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In the first paper of this series (1) data were presented which indicate that the anemia produced in rats by a diet of cow's milk can be relieved by synthetic diets containing less copper than is present in the milk, on the basis of the amounts consumed per rat per day. It was shown that, upon a synthetic ration which was deficient] in vitamins and salts and which contained only a small amount of iron, the regeneration of hemoglobin was strikingly rapid. The level of protein fed per day in the synthetic diet was higher than in the milk upon which anemia was produced. Its quality was different. The speed of hemoglobin production upon this ration may indicate that hemoglobin formation in this type of anemia can be stimulated by means of organic as well as inorganic factors.

Organic factors are of course necessary for the synthesis of the blood pigment. The processes in the animal economy by which the organic nucleus of the *heme* portion of hemoglobin is produced might well be termed *pyrrolegenesis*. The problem of pyrrolegenesis has had a place in the minds of many investigators, but proof of the origin of pyrrole groups within the body has not been forthcoming. The only positive findings in the literature reviewed by us are those of Hirasawa (2) and Matsuoka and Hirasawa (3). These investigators claim that tryptophane and particularly methyl tryptophane stimulate hemoglobin production in various forms of experimental anemia in the rabbit. Cartland and Koch (4), on the other hand, have found that hemoglobin is produced

by growing rats just as effectively upon a diet containing 10 per cent of wheat gluten (corresponding to a consumption of 5 mg. of tryptophane) as upon the same diet + 60 mg. of tryptophane per rat per day. Since the tryptophane was not administered to anemic animals, the findings of Cartland and Koch cannot constitute an adequate criticism of those of the Japanese workers.

The present study of the value of various amino acids in hemoglobin production is based upon two important factors: first, the production of severe nutritional anemia in rats upon a whole milk diet; second, a recognition of the importance of inorganic components such as iron and copper.[1]

Methods

In these experiments albino rats were used. At weaning or shortly after, they were put upon a diet of raw milk. They were kept, either individually or in pairs, in the same cages during the whole course of the experiment. With the development of severe anemia, the milk was modified by the addition of 5.4 mg. of iron as ferric sulfate per quart of milk. This furnished about 0.2 mg. of iron per rat per day, a consumption of 35 cc. of milk being assumed. Although milk containing this amount of iron is ineffective in the cure of the anemic rats, it has been shown that a synthetic diet furnishing daily this small quantity of iron is effective (1).

Three groups of experiments have been carried out in which milk, modified as above, was supplemented by amino acids and fed to the anemic rats. Blood samples were obtained periodically by the usual technique of clipping the tail. Hemoglobin was determined by the Newcomer method, with a calibrated glass plate (1).

Copper-free water (redistilled from glass) was used for the solution of amino acids, etc. and for drinking water for the rats. All the amino acids were ashed in an electric furnace and then analyzed for iron by the method of Kennedy (5) and for copper

[1] For a complete bibliography of the papers of E. B. Hart and coworkers upon the significance of copper in milk anemia see reference (1). This bibliography also contains references (30–32) to papers by H. H. Beard and V. C. Myers, by R. W. Titus, H. W. Cave, and J. S. Hughes, and by H. S. Mitchell and L. Miller upon the value of such metals as manganese, etc.

by the modified Biazzo and ethyl xanthate techniques (1).   The amino acid solutions were made up in small quantities, sufficient for 3 or 4 days, and were kept tightly stoppered in the refrigerator. At the conclusion of the experiments the rats were autopsied and the organs fixed for histological examination.

It may be noted that in the three groups of experiments, carried out at different periods, various individual amino acids were studied at the same time.   They were added in molecular equivalents to portions of the same milk-iron mixture.   Furthermore, after a certain supplement had failed to check the progressive anemia, the effect of another amino acid was studied upon the same animals.   These procedures afford additional controls.

<div align="center">EXPERIMENTAL</div>

First Series of Experiments—In these experiments the value of arginine monohydrochloride as a curative agent in milk anemia was tested.   The progressive anemia could not be checked by the addition of a definite small quantity of iron to the milk.   The results of two typical control experiments out of a group of twelve are shown in Chart I, Experiment A.   When, in addition to this quantity of iron, pure arginine monohydrochloride in amounts of 100 mg. per rat per day was added to the milk, the increase in hemoglobin concentration was very definite.   The degree of hemoglobin regeneration is given in Chart I, Experiments B to H. Experiments G and H indicate that the hemoglobin production was no better with the addition of 200 mg. of arginine monohydrochloride per day than with 100 mg.   Experiments B and F show the recovery from the anemia by the addition of this amino acid after the iron alone had failed.

The arginine monohydrochloride was prepared from hair by a slight modification of the Cox method (6).   The total nitrogen and the amino nitrogen of this preparation agreed with the theoretical.   The $[\alpha]_D^{25°}$ of a 1 per cent solution was $+17.5$, which is a satisfactory criterion of purity (7).   The ash content (probably mainly NaCl) was 0.08 per cent.   0.5 gm. contained 0.036 mg. of iron (or 0.007 mg. of additional iron per rat per day).   The quantity of copper[2] in a 0.5 gm. sample was too small to be deter-

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[2] Sensitive qualitative tests for Mn, Co, and Ni[1] have been made upon solutions of the ash of both arginine and glutamic acid.   These tests were negative upon 10 times the quantity of amino acids fed per rat per day.
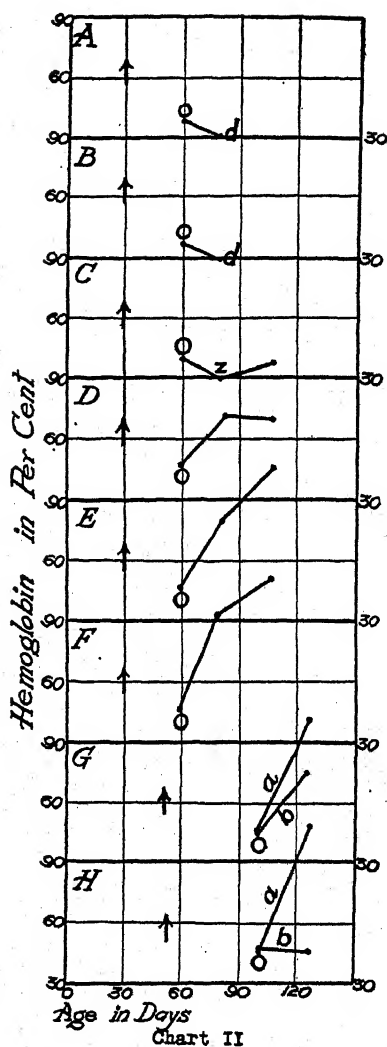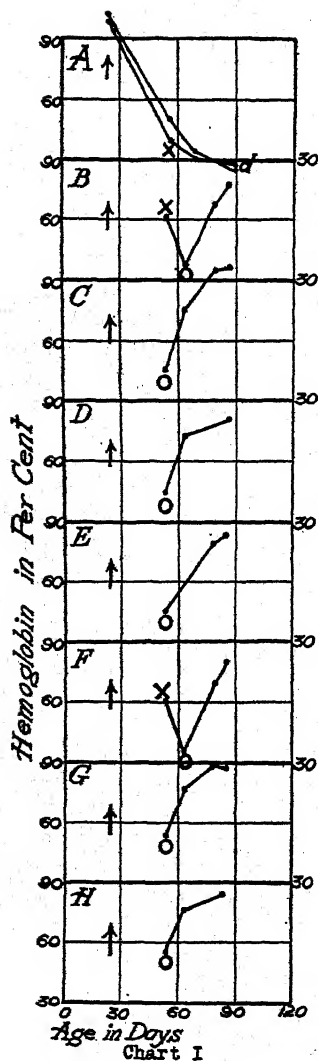
CHART I. The arrows indicate the points at which the rats were placed upon whole milk; crosses, milk supplemented by 0.2 mg. of iron per rat per day; circles, milk supplemented by arginine monohydrochloride, besides the iron.   Experiment A gives data on two control animals (similar to the other ten controls).   In Experiments B to F, 100 mg. of the arginine hydrochloride were used.   Double this quantity was administered in Experiments G and H. d indicates death of animal.

CHART II. The arrows indicate the points at which the rats were placed

mined by the modified Biazzo and modified ethyl xanthate methods (1).

Second Series of Experiments—Chart II shows the value, in terms of hemoglobin regeneration, of alanine, glutamic acid, and proline. These amino acid supplements were fed in quantities molecularly equivalent to 100 mg. of arginine monohydrochloride. In the case of alanine twice the equivalent quantity was also tried. The addition of alanine to the milk-iron mixture was ineffective in stemming the progressive anemia (Experiments A to C, Chart II). Two of these rats died. Strikingly opposite were the results obtained when the same milk-iron mixture was fortified by the addition of glutamic acid. The satisfactory hemoglobin production with this amino acid is shown in Experiments D to F. The data presented in Chart II, Experiments G and H, are upon a group of rats which was put upon milk at a later age than that usually chosen. It took longer to make them anemic. Here, too, the superior value of glutamic acid for hemoglobin building is demonstrated. The alanine hydrochloride supplement in this group was ineffective, while proline was less effective than the glutamic acid. In Experiment C (Chart II), the addition of proline, after alanine had proved valueless, saved the life of the rat, but the hemoglobin production was small.

The alanine was a racemic preparation. The ash content was 0.02 per cent. A 0.5 gm. sample contained 0.008 mg. of iron and was negative for copper. The hydrochloride was prepared by the addition of the calculated quantity of pure hydrochloric acid.

The glutamic acid was purified from a commercial preparation. The latter was decolorized with charcoal, precipitated as the hydrochloride, freed from the HCl by the addition of the theoretical amount of pure NaOH, recrystallized, and washed. The

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upon milk; circles, milk supplemented by 0.2 mg. of iron per rat per day and by amino acids. In Experiments A and B, 42 mg. of alanine were used; in Experiment C, 84 mg. of alanine. In Experiments D, E, F, G,a, and H,a, 70 mg. of glutamic acid were administered. In Experiment H,b, 60 mg. of alanine hydrochloride were fed. Z indicates the supplement was changed from 84 mg. of alanine to a mixture containing 55 mg. of proline. In Experiment G,b 55 mg. of proline were used. d indicates death of animal. The charting of the data on two animals in one space, as in Experiments G and H, is done merely for convenience.
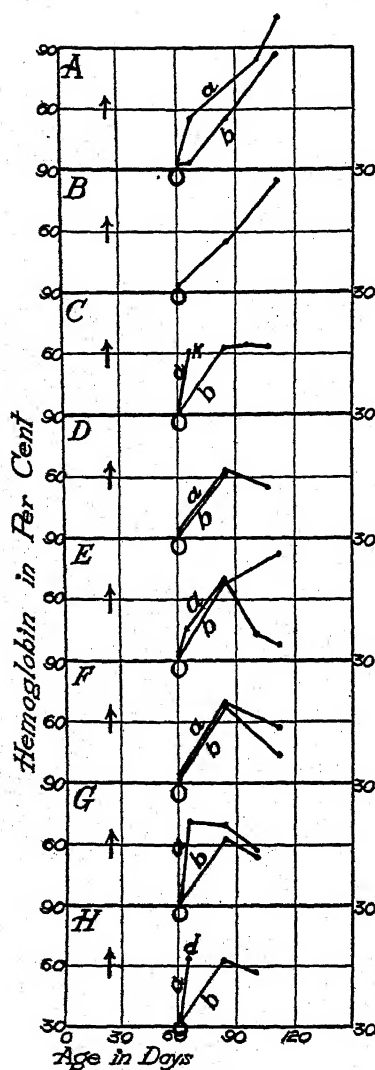
Chart III
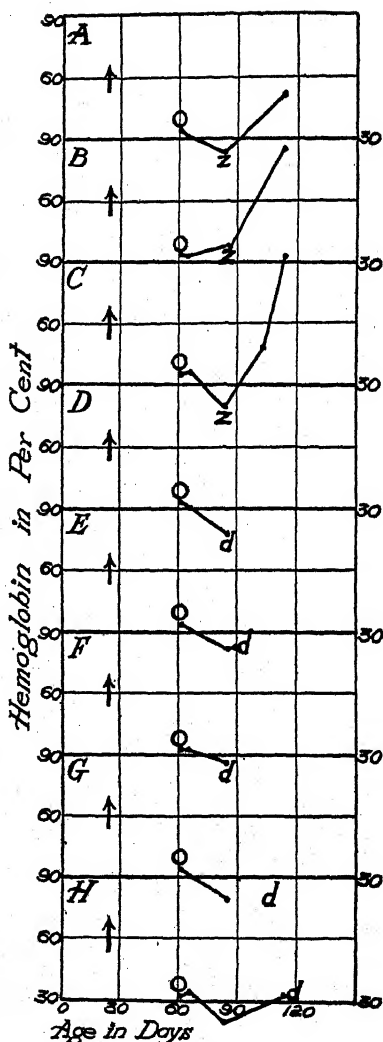
Chart IV

CHART III. The arrows indicate the points at which the rats were placed upon milk; circles, milk supplemented by 0.2 mg. of iron per rat per day and by amino acids.   In Experiments A, a and b, and B, 83 mg. of arginine were administered.   In Experiments C, a and b, and D, a and b, 97 mg. of tryptophane were used.   In Experiments E, a and b, and F, a and b, 74 mg. of sodium hydrogen aspartate were given.   In Experiments G, a and b,

purified product was ash-free (*i.e.* ash less than 0.01 per cent). A 0.5 gm. sample was both iron- and copper-free by the methods of analysis.

The proline preparation was obtained by a butyl alcohol extraction of hydrolyzed gelatin (8). Analyses of optical activity, total nitrogen, and amino nitrogen indicated that the partially purified sample used was only 72 per cent of proline. The mixture was ash-free and contained in a 0.5 gm. sample too little copper for analysis.

Third Series of Experiments—Charts III and IV present the data obtained by feeding the supplements of arginine, tryptophane, sodium hydrogen aspartate, pyrrolidonecarboxylic acid, hydrochloric acid, histidine dihydrochloride, alanine hydrochloride, and sodium hydrogen glutamate. As in the other experiments the amino acids were fed in molecular equivalents and were added to the same milk-iron mixture. The amino acid supplements were fed over longer periods than in the two previous groups of experiments. It is evident that both upon arginine (Chart III, Experiments A and B) and upon sodium hydrogen glutamate (Chart IV, Experiments A to C) the recovery from the severe anemia is notable and continuous during the period of the experiment. Despite the addition of HCl (Chart IV, Experiments A, D, and E), histidine dihydrochloride (Chart IV, Experiments B and F), and alanine hydrochloride (Chart IV, Experiments C, G, and H) to the milk-iron mixture, the anemia became progressively worse and resulted in the death of all these animals, except those changed to sodium hydrogen glutamate (Chart IV, Experiments A to C). The rapid recovery of these rats, which were practically in a state

and H, a and b, 61 mg. of pyrrolidonecarboxylic acid were fed. *k* indicates the animal was killed. *d* indicates death of rat. The charting of the data on two animals in one space, as in Experiments C to H, is done merely for convenience.

CHART IV. The arrows indicate the points at which the rats were placed upon milk; circles, milk supplemented by 0.2 mg. of iron per rat per day and by either HCl or amino acids. In Experiments A, D, and E, 1 cc. of 0.5 N HCl per rat per day (corresponding to the quantity of HCl in 100 mg. of arginine hydrochloride) was given. In Experiments B and F, 109 mg. of histidine dihydrochloride were administered. In Experiments C, G, and H, 60 mg. of alanine hydrochloride were fed. *Z* indicates the supplement was changed to 80 mg. of sodium hydrogen glutamate. *d* indicates death of animal.

of collapse, was most striking in respect both to hemoglobin and growth. The initial stimulus to hemoglobin formation with tryptophane, sodium hydrogen aspartate, and pyrrolidonecarboxylic acid (Chart III, Experiments C to H) was of the same order of magnitude as with the successful amino acids shown in Charts I and II. This initial stimulus was particularly noticeable in the case of the rats fed pyrrolidonecarboxylic acid. Unlike the hemoglobin response in the rats receiving arginine and sodium hydrogen glutamate, the increase in hemoglobin did not continue progressively. In the case of the tryptophane feeding experiments, after the early rise, the hemoglobin was maintained at the level which it had attained. Continued feeding with pyrrolidonecarboxylic acid was accompanied by some falling off in the hemoglobin, while three of the four rats, fed sodium hydrogen aspartate, once again became severely anemic.

Arginine was prepared from the arginine monohydrochloride by the addition of the theoretical quantity of pure NaOH, thus introducing 1 mol of NaCl for each mol of arginine in our preparation.

Tryptophane was a product purchased in the market. The ash content of this preparation was 0.07 per cent. A 0.27 gm. sample contained 0.017 mg. of iron, while the copper content was negative by our methods of analysis.

Sodium hydrogen aspartate was prepared from a commercial sample of aspartic acid by the addition of the theoretical amount of NaOH. The ash content of the aspartic acid was 0.04 per cent. 0.5 gm. contained 0.011 mg. of iron and the copper content was too small to be determined.

The pyrrolidonecarboxylic acid was prepared from glutamic acid by heating (9). This preparation contained 10.65 per cent of total nitrogen, had no amino nitrogen, and its $[\alpha]_D^{25°}$ was − 9.13. These figures are in satisfactory agreement with the theoretical.

The histidine dihydrochloride was purchased in the market. The ash content was 0.11 per cent. The analysis of a 0.25 gm. sample showed 0.024 mg. of iron and a trace of copper (less than 0.01 mg.).

DISCUSSION

All the amino acids, except glutamic acid, which have been used contained traces of iron. The quantities of iron present were so

small, however, that the results cannot possibly be attributed to an increase in the amount of this metal fed.   The amount of iron fed per rat per day was 0.2 mg.   This quantity was insufficient to check the anemia, hence the addition of 0.007 mg. of iron when arginine hydrochloride was administered can hardly be offered as a rational explanation for the stimulus to hemoglobin production which the feeding of this amino acid afforded.   Furthermore, in respect to the copper content, the various amino acids which have been found to stimulate hemoglobin regeneration may be considered as copper-free.   5 to 10 times the quantity fed per day of these amino acids contained too little copper to be determined by the modified xanthate and Biazzo techniques (1).   The suggestion[1] that milk anemic rats cannot be cured unless, besides iron, copper is added to the milk should be modified by the finding that the addition of pure amino acids to milk containing a quantity of iron, insufficient in itself, produces effective hemoglobin building.

The first amino acid which was found to be of value in milk anemia was arginine monohydrochloride.   Although nothing is known concerning gastric physiology in this type of anemia, achlorhydria (10) has long been associated with other forms of anemia.   For this reason it was deemed necessary to find whether or not HCl alone or in combination with an otherwise inert amino acid, such as alanine, would prove to be hemoglobin-stimulative. The various results indicate that, under the present experimental conditions, not only was the administration of HCl alone or in combination with amino acids of no value, but there was found no relationship between the relative acidity of the diet and hemoglobin production.   Arginine and sodium glutamate were just as effective as arginine hydrochloride and glutamic acid.

In these experiments the factor of the quantity of diet consumed is worthy of consideration.   It may well be argued that the addition of certain supplements to milk might stimulate the appetite far more than the addition of others.   Thus rats receiving a palatable and appetizing supplement might consume more milk and the quantity alone (due to the intake of more protein, minerals, etc.) might be responsible for the cure of the anemia.   As has been found in the previous work (1), rats which have become anemic upon a whole milk dietary, consume less milk.   It has been suggested (11) that the reduction in milk consumption is a protec-

tive mechanism to check the progress of the anemia. It is not surprizing that a marasmic animal consumes less diet and in our experiments the anemia became progressively worse in spite of the reduced food consumption. Upon the addition of each of the supplements to the milk, the quantity consumed returned, early in the experiment, to an approximately normal level (30 to 35 cc. per rat per day). This early increase in milk consumption was not maintained, however, in all the experiments. In the case of the rats receiving the HCl supplement, the milk consumption, after a period of 7 to 10 days, again fell to a low level. The rats receiving the alanine hydrochloride supplement maintained their milk consumption at a normal level for a period of 3 weeks, while the anemia was becoming progressively worse. The milk consumption of the rats receiving the proline mixture, which was probably unpalatable, fell to a very low level after the 1st week of feeding. The rats receiving the histidine dihydrochloride and tryptophane supplements consumed their food in approximately equal quantities during the first 3 weeks of feeding, when the histidine rats fell off in their food consumption. In the case of the arginine hydrochloride, arginine, glutamic acid, and sodium glutamate experiments, the food consumption remained at a normal level during the whole period of administration. There was one exception—the rats receiving 200 mg. of arginine hydrochloride per day. After the 1st week of feeding the food consumption of these animals decreased and was no greater than that of the histidine rats. The food intake of the rats fed sodium aspartate and pyrrolidonecarboxylic acid was approximately as great during the whole course of the experiment as the intake of those receiving arginine.

In order to insure complete consumption of the supplement some attempt has been made to add the amino acid supplements to smaller quantities of milk when the consumption fell. This procedure ·was only partially successful since the diets in some cases were not entirely consumed even after the reduction. The hemoglobin levels during the various periods of feeding, as for example during the first 10 days when all the rats consumed the same quantity of milk or at a later period when the arginine, sodium glutamate, pyrrolidonecarboxylic acid, and sodium aspartate rats had approximately the same intakes. are so different

that it is impossible to relate the level of food consumption to the hemoglobin production. This becomes still more apparent by a consideration of the finding of excellent hemoglobin regeneration with a relatively poor milk intake in the case of the arginine hydrochloride rats which received twice the quantity of amino acid. The question as to what effect substances such as the proline mixture would have had upon hemoglobin production had the food intake been normal must, of course, remain open. On the other hand, during the period of normal milk consumption, supplements such as alanine, histidine, and HCl fell definitely into the category of valueless for hemoglobin production in contrast with the other amino acids fed. In the case of histidine too few experiments have been performed thus far to permit more than a tentative statement concerning this amino acid.

From an analysis (12) of the protein nitrogen of cow's milk, it may be calculated roughly (upon the assumption that all the protein is digested to amino acids) that with a milk consumption of 35 cc. per day, a rat was receiving about 780 mg. of amino acids. Thus, the quantities of amino acids added in our experiments on the basis of molecular equivalence of 100 mg. of arginine monohydrochloride, chosen empirically, varied between 5 and 13 per cent of the total amino acids. This would appear to be a relatively insignificant addition. However, if, on the basis of the analysis of casein (13), a rough estimate is made of the percentage increase in individual amino acids entirely different figures are obtained. For example, when 70 mg. of glutamic acid were added to the milk, the increase in this amino acid was about 41 per cent. With the addition of 42 mg. of alanine, the quantity of this amino acid was increased by 290 per cent, while with the addition of 83 mg. of arginine and 97 mg. of tryptophane, these amino acids were increased by about 280 and 830 per cent respectively.

In the light of the above discussion, it is safe to assume that the stimulus to hemoglobin production observed in our various experiments was due to the amino acid supplements. Further work, which is in progress at present, is necessary to elucidate the reason for the difference between the early initial stimulus and cessation of further hemoglobin response with such supplements as tryptophane, sodium aspartate, and pyrrolidonecarboxylic acid, and the continuous, progressive recovery from anemia with arginine, glutamic acid, and their salts. To reconcile the experi-

ments in which anemia was cured by adding sufficient iron or iron and copper to the diet with the present demonstration of hemoglobin production by the addition of pure amino acids to milk containing a low quantity of iron would, at first view, appear to be a most difficult matter. One possibility is that certain organic as well as inorganic substances may exert a beneficial influence locally in the digestive tract of the milk anemic rat, permitting a better or different utilization of foodstuffs already available in the milk. The direct stimulation of blood-forming organs by the two different types of substances, after they are absorbed, is another possibility. In this connection the recent isolation (14) of a crystalline compound of hydroxyglutamic acid and hydroxyproline, effective in the treatment of pernicious anemia, is of great interest. In the nutritional anemia of the rat, however, the most likely possibility appears to be that for the actual synthesis of hemoglobin both iron and organic factors are necessary. As a working hypothesis, the organic supplements which have been found to stimulate hemoglobin production may be considered as sources of pyrrole radicals. Both iron and pyrrole groups are building stones in the synthesis of the blood pigment. The excess of one or the other may be conceived to facilitate their union to form *heme*. Further experiments are in progress at present to test the validity of the theory that organic substances found effective for hemoglobin building exert this action because of *pyrrolegenesis*.

One other observation of interest deserves mention. Rats which had become very anemic upon milk were also stunted. With the inclusion of hemoglobin-stimulating supplements in the dietary growth was resumed. In the sodium glutamate experiments the resumption of growth was particularly striking. These rats very rapidly caught up to and, indeed, surpassed in size many of the others. Thus, although sodium glutamate is not an essential amino acid in the usual sense of the term, the reaction which its administration produced was in every way similar to that described in the studies of amino acid deficiencies.

A careful histological study of the organs of rats used in our work is being made by Dr. George M. Robson of the Department of Pathology. The essential points in the preliminary report upon the histological findings are as follows: The kidneys, heart, lungs, stomach, and spleen of all the rats were essentially normal. Slight parenchymatous degeneration, the significance of which is

uncertain, was observed in the livers of one of the alanine rats, one of the proline rats, and in three of the rats to whom glutamic acid was administered.   The livers of the other rats were normal. It is of interest to note that marked erythropoiesis was found in the spleen and livers of all of the rats fed arginine and in the spleen of one of the glutamic and one of the histidine rats.   This phase of the work will receive further study.

SUMMARY

The relief of milk anemia in rats by the addition of pure amino acids to milk containing a quantity of iron insufficient in itself has been accomplished.

Of the amino acids studied thus far, arginine, glutamic acid, and their salts have proved to be very effective for hemoglobin regeneration.   The recovery from the anemia with these supplements was continuous and progressive.

Tryptophane, pyrrolidonecarboxylic acid, sodium aspartate, and a proline mixture produced an initial increase in hemoglobin, after which the rats were either maintained at the higher level or, as in the case of sodium aspartate, anemia again became severe.

Alanine, alanine hydrochloride, histidine dihydrochloride, and hydrochloric acid feeding was found to be of no value for hemoglobin production in milk anemia.

The various amino acids used were copper-free and contained only insignificant traces of iron.   The possible significance of these findings has been discussed.

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THE FUNDAMENTAL FOOD REQUIREMENTS FOR THE GROWTH OF THE RAT

VI. THE INFLUENCE OF THE FOOD CONSUMPTION AND THE EFFICIENCY QUOTIENT OF THE ANIMAL*

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We have previously (1, 2) described our unsuccessful efforts to devise a synthetic diet for rats on which they will grow at the same rate, and attain the same weight as litter mate or parent controls (of the same sex) fed a natural diet. It was our practice in all these experiments to allow *ad libitum* consumption of food by both the experimental and control animals. Inasmuch as food consumption was recorded on the experimental animals only, the question naturally arose whether the better growth of the controls had been due to a larger food consumption or to the ingestion of growth-promoting factors in the natural diet, not present in the synthetic diet.

This is not a new problem in nutrition. It was discussed at length by Hopkins (3) in connection with his classic experiment showing the remarkable growth-promoting effects of small additions of milk to certain synthetic diets. Mitchell (4, 5), also, has more recently discussed the application of this problem to certain nutrition experiments. Hopkins seems to have proved conclusively, in his experiments at least, that the added natural food increased the velocity of growth out of all proportion to that which could be accounted for by the slightly greater consumption

of energy by the milk-fed animals.   These, and similar observa-
tions, are probably responsible for the tacit assumption by many
physiologists that growth stimulants, at least of dietary origin,
exert their effects primarily on the body cells.

The relation of food consumption to gain or loss of body weight
has been studied in connection with the effects of additions of
mineral salts to the diet, and also in connection with certain
vitamin and amino acid deficiencies.   For example, Mitchell and
Carman (6) showed that the addition of sodium chloride to the
diet of rats and chickens stimulated their growth over that of
control animals, even when the food intake of each test animal
was limited to that consumed by a control.   Mitchell calls this
mode of study "the paired-feeding method."   Using the same
method, Carroll and Mitchell (7) were unable to show that the
feeding of copperas benefited swine on soy bean- or tankage-
containing rations, and Carroll, Mitchell, and Hunt (8) failed to
demonstrate any advantage, for growth, of ferric citrate additions
to a swine ration, or of feeding potassium iodide (9) with the same
ration.

In the vitamin studies, however, Anderson and Smith (10)
demonstrated in vitamin C deficiency with guinea pigs that the
loss in weight cannot be accounted for by lack of food ingestion.
Kon and Drummond (11) showed in vitamin B studies with
pigeons that most of the symptoms of polyneuritis were exhibited
by pigeons receiving vitamin B when their food intake was adjusted
to that ingested by the avitaminotic birds.   Similar results were
obtained by Rose, Stucky, and Cowgill (12) in studies in gastric
motility of dogs suffering from vitamin B avitaminosis.   Jackson
(13), studying the metabolism of tryptophane has shown that
growth failure may be due directly to a deficiency of this amino
acid, as well as to insufficient energy ingestion.   Equalization of
energy intake of experimental and control animals was used in this
demonstration.   Mitchell and Beadles (14) have employed the
paired-feeding method to test the cystine deficiencies of lean beef,
white bread, navy beans, potatoes, milk, and garden peas.   The
results are in line with those of Jackson in indicating that the
addition to a diet of its amino acid deficiency may stimulate
growth even when the total food intake is no greater than that of a
control animal.   It may be pointed out, however, that lack of

uniformity among the different pairs of animals in this study necessitated a statistical study of the results. This is an inherent weakness in the method. It assumes a perfect correlation between food consumption and gain in weight among a population of animals of the same sex. This is an ideal which does not exist. The method not only assumes an "equality of initial capacity to grow and develop on the basal ration" of the pair mates, as pointed out by Mitchell and Beadles (14) but also assumes an equality of food utilization which, as we will show in this paper, is equally important and at present beyond the control of the experimenter.

<center>EXPERIMENTAL</center>

The rather extensive experiments that are summarized in this paper were carried out mostly in 1927 and 1928, although a few were completed in the early part of 1929.

We began our attack on the problem by comparing the growth of rats consuming a synthetic diet *ad libitum* with that of rats consuming an amount of our breeding colony diet adjusted to that consumed by the experimental animals. Animals of only the same sex were compared and food intake adjustments were made each week, dry matter being the basis for the calculations. A preliminary period of 1 or 2 weeks for all the rats on the synthetic diet served as the basis for determining the amount of dry matter as natural diet to be fed the control animals during the 1st experimental week. The synthetic diet consisted of casein (1), butterfat, McCollum's salt mixture,[1] $CaCO_3$, agar, wheat embryo extract on dextrin equivalent to 30 parts of ether-extracted embryo, and tapioca dextrin. Vitamin D was provided for this diet by irradiation. The natural diet used for the controls has been described previously (1). Liquid whole milk was offered or dry whole milk powder incorporated so that one-third of the total dry matter ingested was milk solids. The approximate gross and digestible nutrient composition of the two diets is given in Table I.

We hoped by this method of experimentation, which is analogous to Mitchell's paired-feeding method, to ascertain differences in the effects of the two diets on size and body weight, and on the appear-

[1] McCollum Salt Mixture 185 (see McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

ance and general condition of the animals.   It was regarded as
necessary to continue each experiment for a reasonable length
of time in order to secure this result.   During the progress of the
first few experiments it became apparent, however, that the com-
parisons between animals consuming the same amount of dry
matter would not be valid because of the evident difference in
digestibility of the natural and synthetic diet.   The latter, having
the higher digestibility, was in reality giving better growth.
Digestion coefficients of the diets to be used were required.   We
have determined such coefficients with rats of both sexes, in
groups and singly.   We have been gratified to find that a digestion
experiment can be conducted with rats with great ease and with
remarkably consistent results.   No elaborate equipment is
required.   The McCollum type of feed cup with the hole of such

TABLE I

Major Nutrients in Synthetic and Natural Diets (Dry Matter Basis)

	Protein	N-free extract	Fat	Ash	Nutri- tive ratio
	per cent	*per cent*	*per cent*	*per cent*	
Synthetic diet.....................	18.0	73.0	5.0	4.0	4.80
Natural "	23.3	55.3	12.3	5.0	3.57

size that the animal can just insert its head and neck is sufficient
for accurate food consumption records.   Feces are collected on
filter paper through an elevated floor of 3 mesh screen.   The filter
paper absorbs the urine.   Each test is of 7 days' duration.
   Table II gives a few of the numerous results obtained in deter-
mining the apparent digestibility of the dry matter of various
diets when the procedure described was applied to rats both singly
and in groups.
   After we had established the digestibility of the diets to be com-
pared, it was possible to compare the effects of equal amounts of
digestible dry matter.   We carried out twenty-seven such com-
parisons ranging in length from 5 to 25 weeks.   The average
length was 13 weeks, not counting the preliminary periods.
About 140 animals in all were used.   Although the comparisons
were made in all cases between groups of animals, this fact does

not seem to have vitiated the general results.   In only one experiment were none of the animals in the comparative groups litter mates.   80 per cent of the experiments were strictly litter mate comparisons.

Twenty of the twenty-seven experiments were 12 or more weeks in length.   Although the general results of the shorter and longer

TABLE II

Digestibility in Rats of Various Diets Consumed ad Libitum, with Both Single Animals and Groups of Animals

Diet	No. of rats on test	Sex	Digestion coefficient
Stock food without milk	1	♂	79.54
	1	♂	79.67
	3	♂	80.20
	3	♂	77.40
	1	♀	79.77
	1	♀	80.34
Synthetic diet containing butter fat for vitamin A and wheat embryo extract for B and G	2	♀	91.70
	3	♂	92.60
Whole milk powder only	2		95.96
	4		95.21
Liquid whole milk only	1	♂	92.84
	1	♀	92.62
	3	♀	92.50
Synthetic basal diet plus daily supplement of 600 mg. dry yeast, 500 mg. fresh liver, and 20 gm. fresh lettuce	1	♂	93.45
	1	♂	94.89
	1	♂	92.16
	1	♂	93.08

tests were in accord with those of mean length, the numerical data presented are taken from those experiments having 12 or more weeks' duration.   The first 12 weeks only are considered.   The important data are presented in Tables III and IV, the former giving the results with the male and the latter with the female rats.

From the standpoint of gains it is clear that the animals con-

TABLE III

Comparisons between Effects of Synthetic and Natural Diets on Gains and Food Utilization When Two Groups (Usually Litter Mates) of Male Rats Were Fed Equal Quantities of Digestible Dry Matter

Experiment No.	No. of animals	Mean digestible dry matter consumed per animal	Synthetic diet		Natural diet		Difference in	
			Mean gain	E.Q.*	Mean gain	'E.Q.*	Gain	E.Q.*
		gm.	*gm.*		*gm.*		*gm.*	
1	6	577	147	2.26	185	1.91	+38	+0.35
2	6	629	155	2.80	183	2.09	+28	+0.71
3	8	528	115	3.78	137	3.00	+22	+0.78
4	6	771	135	3.80	157	3.05	+22	+0.75
5	6	644	134	3.53	151	3.02	+17	+0.51
6	6	850	189	2.29	177	2.47	−12	−0.18
7	4	770	156	3.36	167	2.99	+11	+0.38
8	4	742	189	2.34	187	2.37	−2	−0.03
Weighted mean........			149.1		165.3		+16.2	

* Efficiency quotient, see text.

TABLE IV

Comparison between Effects of Synthetic and Natural Diets on Gains and Food Utilization When Two Groups (Usually Litter Mates) of Female Rats Were Fed Equal Quantities of Digestible Dry Matter

Experiment No.	No. of animals	Mean digestible dry matter consumed per animal	Synthetic diet		Natural diet		Difference in	
			Mean gain	E.Q.*	Mean gain	E.Q.*	Gain	E.Q.*
		gm.	*gm.*		*gm.*		*gm.*	
1	3	533	67	6.71	136	2.84	+69	+3.87
2	6	542	106	4.09	116	3.80	+10	+0.29
3	8	463	86	5.27	101	4.05	+15	+1.22
4	4	545	64	8.79	108	4.24	+44	+4.54
5	4	467	64	8.11	87	5.51	+13	+2.60
6	4	712	71	8.53	112	4.82	+41	+3.71
7	6	701	97	5.54	93	5.42	−4	+0.12
8	6	584	79	7.09	94	5.70	+15	+1.39
9	4	682	105	5.09	79	7.37	−26	−2.28
10	4	588	90	5.95	87	6.29	−3	−0.34
11	4	740	108	5.11	115	4.68	+7	+0.43
12	4	761	126	4.55	136	3.97	+10	+0.58
Weighted mean........			90		103.4		+13.4	

* Efficiency quotient, see text.

suming the natural diet made on the average slightly better gains than their litter mate controls consuming the same amount of digestible dry matter in the form of the synthetic diet. Six of the eight experiments with the male rats and eight of the twelve experiments with the female rats showed this advantage for the natural diet. In only two tests, one for male rats and one for females, was the synthetic diet apparently distinctly better than the natural. There was no significant difference between them in the remaining four tests, one with the males and three with the females. It is debatable whether the differences in gains noted are to be considered growth differences. Perhaps this is true for Experiment 1 with the males (Table III) and Experiments 1, 4, and 6 (Table IV) with the females. At the same time it should be borne in mind that the data presented are mean results and do not indicate that each animal in one group made the same superior gain over each animal in its corresponding group. Besides the general greater gains for the animals consuming the natural diet, there was noted a superior physical appearance for these animals. This was true even for those comparisons where no differences in gains were made. Since the less thrifty appearing animals always received the synthetic diet, this observed difference, at least, seems attributable to differences in composition or constitution of the two diets. Table I shows that the natural diet contained a slightly higher proportion of protein and a considerably higher proportion of fat.

Although the mean effects noted favor the natural diet, the really striking fact brought out in these comparisons is the great variation between different groups consuming the same kind and quantity of food during the same period of time. Unfortunately the data secured do not give as many comparisons as would be desired between animals consuming exactly the same quantity of digestible dry matter of the same composition. It will be recalled that there was no restriction of food consumption with the group offered the synthetic diet, so that this varied considerably from group to group. A few instances occurred where the desired comparison may be made. Experiments 2 and 5, and 4 and 7 with the male rats (Table III), and Experiments 2 and 4, 3 and 5, 8 and 10, and possibly 11 and 12 with the female rats (Table IV) are instances of this sort. The variations noted in these comparisons,

which are borne out by the experiments as a whole, indicated clearly that some other important factor besides food consumption was responsible for the results obtained.   Numerous calculations from the data pointed strongly to variations in food utilization as the probable vitiating factor.   This is supported by the figures in the fifth and seventh columns of Tables III and IV which we may refer to as the efficiency quotient.[2]   This figure represents the digestible dry matter consumed in gm. per gm. of gain in weight per 100 gm. of body weight.   The calculation is actually made as follows:

$$\text{Efficiency quotient} = \frac{\dfrac{\text{Digestible dry matter consumed}}{\text{Gain in weight}}}{\text{Mean weight during experiment}} \times 100$$

The great variation in the efficiency quotients calculated for the experiments summarized in Tables III and IV showed that comparisons such as we sought between the nutritive value of two diets could only be made valid when the animals used had the same efficiency of food utilization for gain in weight.   Obviously this could be determined only by preliminary trials.

To this end we undertook a series of experiments in which young animals at weaning (28 days of age) were placed in individual cages and allowed *ad libitum* consumption of a high protein (35 per cent casein), high fat (24 per cent) basal synthetic diet[3] containing vitamins A and D, dried yeast[4] being fed separately each day.   At the end of 6 to 8 weeks the efficiency quotient was calcu-

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[2] We have presented this method of calculating the efficiency of food utilization in a previously published study, Palmer, L. S., and Kennedy, C., *Proc. Soc. Exp. Biol. and Med.*, **26**, 427 (1929).

[3] This diet consisted of purified casein 35 parts (see Palmer, L. S., and Kennedy, C., *J. Biol. Chem.*, **74**, 597 (1927) for method of purification), corn-starch 37 parts, rendered, filtered butter fat from June butter 9 parts, leaf lard 15 parts, salt mixture 4 parts (see Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **34**, 131 (1918), control salt mixture without lactose). The ration without the fat was irradiated for 30 minutes with an Alpine Sun lamp at a distance of 1 foot before incorporating the melted fats. 600 mg. of dried yeast were fed separately in tablet form in the preliminary period.

[4] Dried yeast from the Northwestern Yeast Company, Chicago, was used throughout the study.

lated for each animal, and animals of the same sex and having nearly the same efficiency quotient used for the subsequent comparative study on the same basal diet.   Table V shows the results of three such comparisons between yeast and alcoholic wheat embryo extract as sources of water-soluble vitamins.   These comparisons were made during the period February to July, 1928. The food consumed by the animal fed wheat embryo extract was offered to the animal fed the dried yeast.   2 gm. daily of embryo extract on dextrin and dried yeast, respectively, were fed.   The embryo preparation was equivalent to about 2.5 gm. of ether-extracted embryo.

TABLE V

Yeast versus Wheat Embryo Extract with Rats with Same Quotient and Equal Food Intake of Both Basal Synthetic Diet and Supplement

Comparison No.	Sex	Preliminary Q	Duration of test		Gain during experimental period
			Preliminary	Experimental	
			days	*days*	*gm.*
1* a	♂	2.544	35	98	130
b	♂	2.485	35	98	128
2 a	♂	2.00	35	91	71
b	♂	2.15	35	91	108
3 a	♂	2.40	35	77	56
b	♂	2.25	35	77	52

a = wheat embryo extract; b = yeast.
* Litter mates.

In only one of the three "paired-feeding" tests was the yeast superior to the embryo extract as a source of growth-promoting vitamins.   Inasmuch as a great deal of evidence exists to indicate that the rat (b) in each of these tests would have made much better growth in the time allowed had he been permitted to consume the basal diet *ad libitum*, the inference seems to be that the rate of gain in weight of animals having the same ability to utilize their food nutrients is governed more by the amount of food ingested than by its vitamin content.   This conclusion is contrary to the findings of Hopkins (3), discussed earlier.

A careful study of the implications of these results led us to apply this method of study to other alleged growth stimulants. At the time these studies were begun, Osborne and Mendel (15) had reported the remarkable growth rate of male rats fed supplements of yeast and liver, yeast and lettuce, etc., with a synthetic diet; Mendel and Cannon (16) had reported additional data for male rats and similar data for females fed like diets. The possibility is suggested in the former paper that these results are due to the presence of unrecognized dietary essentials in the supplements fed.

Griffith and Graham (17) have studied the effect of yeast and yeast plus liver extract on the food consumption, food utilization (calories per gm. of increase in weight), and growth of male rats. Their results, given in terms of averages, lead them to the conclusion that yeast increases the food utilization but that the liver extract exerts its effect on growth through stimulation of the appetite. The data so far available do not indicate whether the comparisons were made between individual animals or between groups of animals. Restricted food intake was employed for the comparisons but the inherent efficiency of the animals in food utilization does not seem to have been taken into account. However, Griffith (18, 19) seems to recognize clearly that efficiency of food utilization (rate of growth per gm. of food consumed) must be taken into account in studies involving the effect of diet on growth. His method of expressing this fact is the usual one long employed in farm animal feeding trials, namely, a simple economy of gain based either on the total nutrient or energy consumption. Several investigators have employed such calculations in interpreting nutrition experiments with rats (see Smith and Carey (20), Levine and Smith (21), Hitchcock (22), Evvard, Nelson, and Sewell (23)). This method does not allow for the food cost of maintenance, which becomes increasingly important as growth proceeds. Hopkins (3) and Macallum (24) calculated the energy cost per unit of gain and also the energy intake per unit of body weight, but made no attempt to combine the results. The size of the animal and its maintenance requirement is taken into consideration in our method of calculation which attempts to determine the efficiency of gain per unit of body weight.

The growth stimulants studied by our procedure have included

yeast, liver, lettuce, and carrots.   Between 160 and 170 animals have been employed.   We first determined the effects of lettuce and liver on the digestibility of a synthetic basal diet to which a yeast supplement was given.   Although it seemed improbable that the high digestibility of this basal diet could be materially improved by these supplements, it was necessary to determine whether the growth stimulation could be due in any measure to such an effect.

Using one set of animals for the lettuce study and another for the liver we found that lettuce fed in the quantities reported by Osborne and Mendel (15) as the sole extra supplement (40 gm. of fresh lettuce daily) depressed the apparent digestibility of the dry matter from 93 to 90 per cent.   Fresh liver as the sole extra supplement (10 gm. daily) also decreased the apparent digestibility of the dry matter, but to a less extent, *i.e.* from 93.2 to 91.2 per cent.   Obviously the effects of these supplements cannot be attributed to an increase in the digestibility of the rations containing them.

The next series of experiments were for the purpose of determining the effects of a liver + lettuce + yeast supplement on the efficiency quotient.   One assumption that was tested was that if two animals with the same efficiency quotient are given the same quantity of supplements daily, but the basal diet of one is restricted to a definite proportion of that consumed by the other, any effects of the supplements on the efficiency quotient would be manifested on the efficiency quotient of the animal fed the restricted diet, even though the two animals did not gain the same amount.   In some of these tests the supplements fed amounted to 40 gm. of fresh lettuce and 10 gm. of fresh liver[5] in addition to 600 mg. of dried yeast.   In other tests these amounts were reduced to 20 gm. of lettuce and 5 gm. of liver.   The period of lettuce and liver feeding lasted 21 days and was preceded by a preliminary period of 28 to 49 days.   The latter was for the purpose of selecting the animals of uniform efficiency quotient for the later comparisons. Yeast was the only supplement fed during the preliminary period. An interval of several days between the preliminary and liver-lettuce feeding allowed the animals to become accustomed to the

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[5] Head lettuce and cow's liver were employed.

new supplements. In the comparative tests of *ad libitum* consumption and restricted food intake the adjustments of food consumption of the latter were made daily.

A portion of the lettuce-liver feeding period was also a simultaneous digestion experiment for each animal in order that the

TABLE VI

Influence of Liver and Lettuce Supplements on the Digestibility with Various Comparative Levels of a Basal Synthetic Diet

Rat No. and sex	Intake of basal diet.	Daily intake		Coefficient of digestibility of dry matter		
		Liver	Lettuce			
		gm.	*gm.*	*per cent*		
♂3025	*Ad libitum*	10	40	91.91		
♂3026	" "	10	40	92.44	92.17 average	
♂3011	" "	5	20	90.80		
♂3013	" "	5	20	89.70		
♂3030	" "	5	20	90.57		
♂3020	" "	5	20	95.25		
♀3018	" "	5	20	94.73		
♀3016	" "	5	20	93.40		
♀3035	" "	5	20	90.62	92.15	"
♀3036	¾ feed	5	20	92.04		
♂3012	⅔ "	5	20	91.88		
♂3014	⅔ "	5	20	93.38		
♂3029	⅔ "	5	20	92.38		
♀3015	⅔ "	5	20	92.60	92.46	"
♂3022	½ "	10	40	90.48		
♂3023	½ "	10	40	90.78	90.63	"
♂3021	½ "	5	20	94.50		
♀3017	½ "	5	20	96.10	95.30	"

efficiency quotient could be calculated on the basis of digestible dry matter consumed. Table VI shows the effects on digestibility of full and restricted feeding with constant intake of the supplements. The supplements of liver and lettuce appeared to depress the digestibility of the restricted diets only when the larger quantities of supplements were fed.

Table VII shows the effects of the intake of the liver and lettuce supplements on the efficiency quotient when the diet was restricted, the comparison being between the preliminary and experimental periods for each animal.   In no case did the lettuce and liver lower the efficiency quotient (increase the efficiency) when the basal diet was restricted.   However, in five of the eight comparisons on full feed (Experiments 1, 3 to 5, and 6) the introduction of liver and lettuce seems to have increased the efficiency of food utilization.

TABLE VII

Effect on Efficiency Quotient of Adding Liver and Lettuce to a Basal Synthetic Diet and of Restricting the Intake of the Basal Diet

Experiment No.	Rat No. and sex	Comparison, full feed with	Preliminary E.Q.*	Experimental E.Q.*
1	♂3011	Full feed	1.75	1.64
2	♂3013	" "	1.85	1.92
3	♂3020	" "	2.46	1.72
4	♂3025	" "	2.54	1.33
5	♂3026	" "	2.29	1.78
6	♂3030	" "	1.98	1 23
7	♀3016	" "	2.56	3.89
8	♀3035	" "	1.99	1.97
9	♀3036	$\frac{3}{4}$ "	1.90	4.04
10	♂3012	$\frac{2}{3}$ "	1.62	11.30
11	♂3014	$\frac{2}{3}$ "	1.99	7.98
12	♂3029	$\frac{2}{3}$ "	2.00	1.99
13	♀3015	$\frac{2}{3}$ "	3.21	7.37
14	♂3021	$\frac{1}{2}$ "	2.47	6.71
15	♂3022	$\frac{1}{2}$ "	2.08	3.49
16	♂3023	$\frac{1}{2}$ "	2.38	3.29
17	♀3017	$\frac{1}{2}$ "	2.24	Did not gain

* Efficiency quotient, see text.

The data are not available, however, to decide whether this is an actual effect of the supplements or a natural trend due to advancing age.

In Table VIII the efficiency quotients of the paired animals are compared during the experimental period when the food consumption of one animal of each pair served as the basis for calculating daily the restriction of diet for its experimental mate, the initial efficiency quotient of the two animals being alike or similar.

Where a slight difference did exist, the advantage was usually given to the animal to be put on restricted diet; *i.e.*, the diet of the animal with the lower efficiency quotient was restricted. This method of presenting the results likewise fails to show that the supplements in question favorably affected the utilization of food. Their growth-stimulating effects seem to be due to their effects on food consumption.

These results support the conclusion drawn by Griffith and Graham (17) using a liver extract, although their method of experi-

TABLE VIII

Comparison of Efficiency Quotient of Animals with Same Initial Efficiency Quotient When the Basal Diet of One Animal is Restricted to a Definite Proportion of the Other, Both Animals Ingesting Equal Amounts of Liver, Lettuce, and Yeast

Experiment No.	Initial E.Q.*			Comparison, full feed with	E.Q.*, full feed	E.Q.* restricted feed
	a†	b†	Mean			
1	1.99	1.90	1.95	¾ feed	1.97	4.04
2	1.75	1.62	1.68	⅔ "	1.64	1.99
3	1.85	1.99	1.92	⅔ "	1.92	7.98
4	1.98	2.00	1.99	⅔ "	1.23	1.99
5	3.26	3.21	3.23	⅔ "	3.64	7.37
6	2.54	2.08	2.31	½ "	1.33	3.49
7	2.29	2.38	2.34	½ "	1.78	3.29
8	2.46	2.47	2.47	½ "	1.72	6.71

* Efficiency quotient, see text.

† a represents the animal later kept on full feed; b, the animal later kept on restricted diet.

mentation did not permit comparisons between animals with the same initial efficiency.

Osborne and Mendel (15) and Mendel and Cannon (16) have reported that male rats frequently gain over 5 gm. daily and female rats over 3 gm. daily during the period of rapid growth when fed a basal synthetic diet containing 35 per cent protein and 24 per cent fat which is supplemented by 250 mg. of dried yeast, 500 mg. of fresh liver, and 20 gm. of fresh lettuce daily. It seemed worth while to study the efficiency quotient of numerous animals of both sexes during the period of rapid growth on such a diet. It

was purposed to compare the effects of the supplements mentioned in various combinations and also the effects of other natural foods, such as fresh carrots.   Data were also sought on animals fed our own stock diet for our breeding colony.   For these purposes we adopted a standard procedure of starting all animals at 60 gm. of weight after weaning and continuing the experiment for 42 days. Growth during this period is expressed essentially as a straight line.   The calculation of the efficiency quotient for such a standard procedure may thus be considered an index of the animal's efficiency or its efficiency index.

Table IX shows the summarized data of a limited number of

TABLE IX

Influence of Various Supplements on Daily Gain in Weight and on the Efficiency Index of the Animal (Dry Matter Basis)

No. of rats	Sex	Supplements	Average daily gain		Efficiency index	
			Mean	Range	Mean	Range
			gm.	*gm.*		
40	♂	Yeast, lettuce, liver	4.78	3.70–5.66	1.50	1.19–2.33
24	♀	" " "	3.18	2.07–4.28	2.40	1.83–4.07
11	♂	" only	3.26	1.72–4.25	2.48	
4	♂	" lettuce	3.86	3.31–4.55	1.62	
7	♂	" liver	4.00	3.50–4.12	1.49	
4	♂	" carrots*	4.81	4.07–5.19	1.32	
4	♂	Stock food and milk	4.18	3.96–4.40		

* Carrot test for 4 weeks only.   All others for 6 weeks.

animals collected in the manner described.   The dry matter only, not the digestible dry matter, was used in these calculations.

In the trials with yeast + lettuce + liver, 50 per cent of the male rats gained more than 5 gm. daily (on the average) and 70 per cent of the females exceeded 3 gm. average daily gain.   Our data seem to offer for the first time an explanation of this sex difference in growth rate, namely, the difference in the efficiency indices of the animals.   The ratio of ♂ : ♀ average daily gain for the first two groups of animals shown in Table IX is 1.503 which is very close to the ratio of $\frac{1}{\text{Efficiency index}}$ for the ♂ : ♀, namely 1.6.

A careful study of the individual variations in response to the
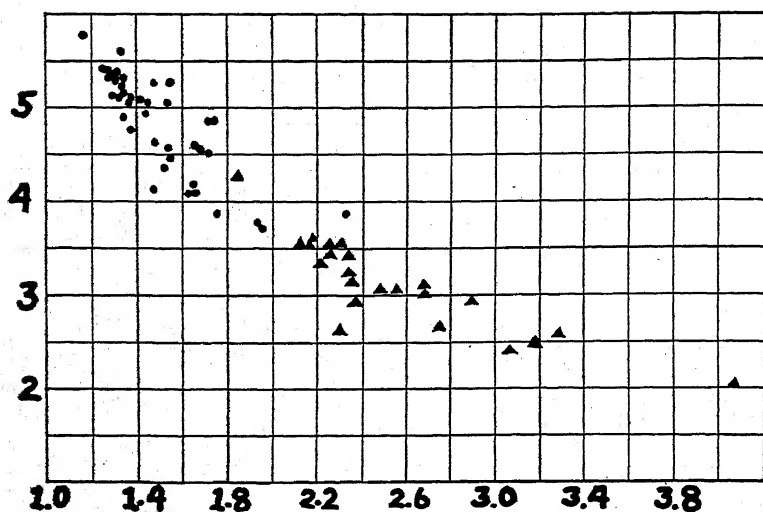
FIG. 1. Chart showing the relation between average daily gain in gm. (ordinates) and efficiency index (abscissa), in terms of dry matter consumed per gm. of gain per 100 gm. of body weight.   The dots represent ♂ and the triangles, ♀ rats.
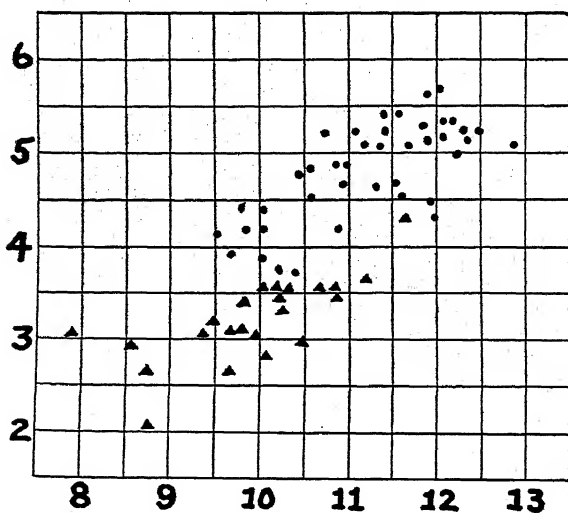
FIG. 2. Chart showing the relation between average daily gain in gm. (ordinates) and average daily dry matter consumed in gm. (abscissa). The dots represent ♂ and the triangles, ♀ rats.

same diet seems to point clearly to a variation in the efficiency index as the major cause. Fig. 1, showing the relation between the average daily gain and the efficiency index of each of the forty male and twenty-four female rats fed supplements of yeast + lettuce + liver (Table IX), seems to point clearly to a close relation between the rapidity of growth and the efficiency index. There seems to be, in fact, a closer relation between these values than between the average daily gain and average daily food consumption of the same animals. The latter relationship is shown in Fig. 2. A statistical treatment of these data is postponed until a larger number of animals of both sexes have been tested.

<p style="text-align:center">DISCUSSION</p>

The experiments presented in this paper seem to us to present numerous problems of great importance for nutrition investigations. This is particularly true of all studies designed to measure the effect of food upon growth or in which the effect of food upon growth is used as a quantitative measure of some nutritive factor. Irwin, Brandt, and Nelson (25) have recently shown that the individual variation in gain in weight of rats in response to vitamin additions to the diet are so great that relatively large numbers of individuals are required in order to secure statistically significant results. These differences and others of a similar nature in other types of feeding experiments are evidently due to two main factors, (1) the food intake, and (2) the efficiency of food utilization. It is obvious that all of the experiments in the literature purporting to measure the effects of food upon growth are influenced by both of these factors. It is impossible to know at present what their relative importance was in any given experiment. It is not improbable that many results that have been interpreted as measuring differences in the nutritive value of two foods or two diets were merely measures of the differences in the average efficiency of the animals employed.

It is at present customary in nutrition studies to assume that individual variations are reduced to negligible proportions by using litter mates. Such an assumption is not accepted by geneticists unless the parents are homozygous for the factor in question. It is, of course, outside of the realm of probability that many parents of rats used for nutrition studies in our various

laboratories are homozygous for efficiency of food utilization.   In fact, no published data are available to indicate whether such a physiological characteristic is inheritable or what its mode of inheritance may be.   A study of these problems is imperative, if the factor of food utilization is to be brought under control.   If it should prove possible to breed a strain of laboratory animals having essentially the same efficiency, comparisons of the effects of different diets on growth would be of much greater significance than at the present time.   The labor of carrying out such experiments would be reduced to a minimum because of the reduction in number of animals required.   The paired-feeding. method, which is undoubtedly a step in the right direction, could then attain its full usefulness and would not require, as do all other methods, statistical treatment.

Many other lines of investigation are suggested by these studies when one appreciates more fully the importance of the difference between individual animals in their efficiency of food utilization. Many of these problems present difficulties of considerable magnitude and are not to be undertaken lightly.   One of these is the cause of the sex difference in efficiency and the possible influence of desexing upon it.   All problems related to the effect of dietary and environmental influences upon efficiency are complicated by the unknown efficiency of the animal before putting it upon experiment.   Although the solution of such problems would be greatly simplified if the animals were homozygous for the factor, we have reason to believe that some progress may be made in this direction before such an ideal is attained.

<div align="center">CONCLUSIONS</div>

In a study of synthetic *versus* natural diets, appreciable differences in the average growth curve of comparative groups of rats of the same sex practically disappear when the food intake is equalized to an equal intake of digestible dry matter.

Large variations in growth between comparative groups of rats of the same sex consuming a synthetic diet *ad libitum* or fed restricted amounts of a natural diet are due to differences in efficiency of food utilization.

Yeast and wheat embryo extract exert the same effect on growth as sources of vitamins B and G when tested with animals

having equalized food intake and the same predetermined efficiency of food utilization.

The so called stimulating effects of fresh lettuce, fresh liver, and of carrots, in various combinations with yeast, on growth appear to be due solely to effects on food consumption when studied by this method.

The efficiency of food utilization for growth is best expressed as the efficiency quotient, gain per gm. of food per 100 gm. of body weight.

The efficiency quotient may be used to determine the efficiency index of an animal when standardized as to size of animal employed, time of duration of test, and constancy of diet.

There is a marked difference in the efficiency index of male and female rats, which appears to be a major factor in determining the difference in growth rate of the two sexes.

Individual variation in efficiency index is a major, if not the controlling cause of individual variation in gain in weight of animals on the same diet.

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A STUDY OF ELECTROLYTE EQUILIBRIUM IN THE BLOOD IN EXPERIMENTAL ACIDOSIS

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INTRODUCTION

The purpose of the experiments included in this paper was to test quantitatively the response made by the mammalian organism to the sudden intravenous injection of massive doses of hydrochloric acid. Normally the acid-base condition of the organism as measured by the bicarbonate and pH of blood or any other two related variables is very constant. Pathologically, as in diabetic and nephritic acidosis, the change in the acid-base condition is produced so gradually that a steady state exists. By producing a rapid, maximum disturbance in the acid-base condition of the animal and studying the subsequent course of the return of the blood toward normal, it was hoped that the factors concerned with the physiological regulation of the acid-base and salt balance could be evaluated.

Numerous authors have investigated the effects of alteration of the acid-base equilibrium of blood *in vitro*. Laboratories prominent in this work have been those of Henderson and Van Slyke (1–7) in this country and Haldane and Barcroft in England. Henderson and Van Slyke have introduced the nomographic method of showing the quantitative relationship between the serum constituents, H_2O, pH, $BHCO_3$, Cl, carbon dioxide and oxygen tensions, base bound by protein and total base, and the cell constituents, H_2O, pH, $BHCO_3$, Cl, base bound by hemoglobin, and total base. In our work, the path taken during and following the injection of acid was determined for as many of these dependent variables as was experimentally feasible.

A few authors have investigated the effects of the injection of acid *in vivo*. Their work deals mainly with serum or whole blood analyses and not with the equilibrium between cells and serum. Since it was to be the purpose of our work to test *in vivo* the hypothesis concerning electrolyte distribution in the blood deduced from experiments *in vitro*, these references are only indirectly relevant.

In 1902, Hamburger (8) injected into the jugular vein of a horse 2 liters of isotonic sodium sulfate solution containing 40 cc. of sulfuric acid (20 cc. of concentrated sulfuric acid + 20 cc. of water). He took blood samples before the injection, 7 minutes after, and 15 minutes after. In the three samples he found the water per cc. of serum to be 0.925, 0.922, and 0.923, showing a fall despite the large introduction of fluid, with a return toward normal. He also noted that the titrable chloride in the serum and the property he called "alkalescenz" fell in the 7 minute sample and returned practically to normal in the 15 minute sample.

Austin and Cullen (9) did intravenous injection experiments in dogs with hydrochloric, phosphoric, and lactic acids. They found considerable reduction in bicarbonate and pH in the case of hydrochloric and phosphoric acids. In the case of lactic acid, the bicarbonate was reduced but the pH remained constant.

Koehler (10) studied the effect of acid and base ingestion on the acid-base balance in patients treated for chronic lead poisoning. These patients were treated for a long period of time with phosphoric acid, ammonium chloride, calcium chloride, and diammonium phosphate. The resultant acidosis showed a pH of from 7.2 to 7.3 and a loss in body weight and dehydration.

Other authors have done work along associated lines as listed below: (1) Durr (11) found that an acid diet led to a more acid urine, lower alveolar carbon dioxide tension, increased respiration, and a higher metabolism. (2) Haldane (12) produced an acidosis by the feeding of calcium chloride and ammonium chloride. He noted a fall in the alveolar carbon dioxide and a rise in ammonium and phosphate excretion. Most of the calcium in the case of calcium chloride ingestion was lost through the feces, while the chloride was completely absorbed. (3) Campbell (13) found that intravenously injected hydrochloric acid decreased the oxygen consumption and carbon dioxide production. (4) Greenwald and Lewman (14) studied the effects of the injection of hydrochloric and lactic acids. There was a reduction in bicarbonate and pH, the lowest being 6.8 at 20°. (5) Taistra (15) found that ingestion of hydrochloric acid reduced the carbon dioxide capacity of serum by 11 to 28 volumes per cent. (6) The results of Haggard and Henderson (16) on the effect of the intravenous injection of hydrochloric acid on the plasma carbon dioxide and pH will be discussed later. (7) Loevenhart, Gamble, Ross, Tisdall, and others have done related work.

Methods

Dogs under barbital anesthesia were used throughout the work. Control experiments were run to determine the effect of the anesthetic on the factors studied. The femoral vein on the left side and the femoral artery on the right were cannulated—the former for injection, the latter for withdrawal of samples of arterial blood for analysis. Twenty-two injection experiments, two controls, and three *in vitro* experiments were performed. An amount of normal hydrochloric acid sufficient to approximately halve the bicarbonate concentration of the blood if the acid were uniformly distributed throughout the body was injected. The quantity injected was calculated to be 5 times that which would be necessary to halve the blood bicarbonate *in vitro*. The blood volume was assumed to be one-twelfth of the body weight for this calculation. The acid was injected at a uniform rate over a period of 3 minutes. More rapid injection was usually followed by immediate death. This dosage was $3\frac{1}{3}$ cc. of N hydrochloric acid per kilo of body weight. In some of the later experiments only two-thirds of this dosage was used. On the assumption that Hamburger's horse weighed 330 kilos, his dosage is to ours as 3 is to 8. In the first series of our experiments, in which the full dosage was used, there were five deaths during the injection and ten successful injections. With the two-thirds dosage there were three deaths during injection and four successful injections. In the successful experiments death usually ensued in from 8 to 12 hours with a maximum of about 21 hours. The control animals lived about the same length of time, which indicates that the barbital rather than the acid was the cause of death.

Blood samples were drawn before, sometimes during, and at various intervals after the injection. The maximum number of samples drawn in one experiment was twenty-six. The blood for analysis was drawn directly into centrifuge tubes under oil. It was then defibrinated, the oil replaced by paraffin, and centrifuged. The serum and cells were separated and kept in small sampling tubes over mercury in order that exchange of gases with the air would be prevented. All cell analyses were done by weight; all serum analyses by volume. To keep cell respiration at a minimum, 1 drop of 0.1 N potassium cyanide was added for each

10 cc. of blood, making the concentration of potassium cyanide about 0.5 mm.

The pH of the serum was determined electrometrically at 38° by the use of the quinhydrone electrode. The standard of reference used in the calculation of pH values was 0.1 N hydrochloric acid, its pH being assumed to be 1.08. In many instances colorimetric determinations by the method of Hastings and Sendroy (17) were also made. In the course of the experiments colorimetric and quinhydrone pH determinations were made on twenty-four specimens of normal dog blood and thirty-seven specimens of blood following the injection of acid. The colorimetric observations on the normal blood averaged 0.04 pH higher than the corresponding quinhydrone determinations. On the acid blood the colorimetric determinations were on the average 0.14 pH higher than the corresponding quinhydrone determinations.

Carbon dioxide analyses of serum and cells were made by the Van Slyke blood gas apparatus. The serum carbon dioxide analyses were made in the usual manner, but the correction factors used were 1.014 times the original factors. This was found by determining the reabsorption factor i for a standard sodium carbonate solution which was found to be 1.028 instead of the original 1.014. These factors are almost identical with those published by Van Slyke and Sendroy after this work was done.

In analyzing the cells for CO_2 the following procedure was adopted. A known weight of cells was laked, in CO_2-free water containing saponin, in the blood gas apparatus before the solution was acidified. The total volume of reagents plus cells was 7.0 cc. The factors used in calculating the CO_2 of the cells and the corresponding temperatures are given in the accompanying tabular matter.

Temperature °C.	Factor
20.0	0.1294
21.0	0.1285
22.0	0.1276
23.0	0.1267
24.0	0.1259
25.0	0.1250
26.0	0.1242
27.0	0.1233

Chloride analyses were made by the wet ashing method of Van Slyke (18).

Water determinations were made by evaporating to dryness at 110°. In the experiments in which the water content of serum was not determined, it was assumed for the purposes of calculation to be 0.940.

As a further check on water changes refractive index determinations of the serum were made with the Bausch and Lomb Abbé refractometer.

Records of respiration were obtained before, during, and after the injection by taking pneumographic tracings. In the last two experiments the blood pressure was recorded.

Calculations

Concentrations of total CO_2, $BHCO_3$, and Cl of both serum and cells have been expressed as millimols per kilo of water.

In the calculation of the CO_2 tensions of the blood, designated p_{CO_2}, the Henderson-Hasselbalch equation has been employed.

$$pH_s = pK' + \log \frac{[CO_2]_s - [H_2CO_3]_s}{[H_2CO_3]_s}$$

where pH and $[CO_2]_s$ are known and pK' has the value of 6.13 in serum.

$$p_{CO_2} = \frac{760 \times 22.4}{0.56 \times 1000} [H_2CO_3]_s = 30.33 [H_2CO_3]_s$$

$$[H_2CO_3]_c = \frac{0.54 \times 1000}{760 \times 22.4} p_{CO_2} = 0.318 \, p_{CO_2}$$

$$[BHCO_3]_c = [CO_2]_c - [H_2CO_3]_c$$

$$[BHCO_3]_s = [CO_2]_s - [H_2CO_3]_s$$

Results

1. Effect of Injection of Acid on the Acid-Base Path of Blood

In the first series of eight experiments, it was desired to obtain as many samples of blood during and after the injection as possible in order to know at just what time the maximum effect was ob-

tained and what was the maximum amount of acid capable of being injected. In Hamburger's work (8) the first sample was taken 7 minutes after the injection. As will be pointed out later,

TABLE I

Experiment I. Acid-Base Relations in the Blood of a Dog

Date, April 21, 1927; dog weight, 8.8 kilos; anesthetic, 2.7 gm. barbital; injection, 32 cc. N hydrochloric acid; death, 10 hours after injection.

Sample No.	Time		Amount of blood	pH, colorimetric	Total [CO$_2$]	[BHCO$_3$]	[H$_2$CO$_3$]	CO$_2$ tension
			cc.		mM per kg. H$_2$O	mM per kg. H$_2$O	mM per kg. H$_2$O	mm. Hg
1	−2	min.	8	7.42	24.43	23.24	1.192	36.1
2	−1	"	8	7.45	23.86	22.77	1.090	33.1
3	+0.75	"	8	7.16	20.74	18.97	1.770	53.3
4	1.5	"	9	7.12	12.67	11.49	1.176	35.6
5	2.25	"	8	6.97	7.99	6.98	1.009	30.6
6	3.50	"	10	6.92	3.89	3.35	0.542	16.4
7	5	"	7	7.07	6.27	5.62	0.646	19.6
8	7	"	5	7.13	9.40	8.54	0.855	25.9
9	11	"	6	7.17	10.80	9.89	0.906	27.5
10	15	"	7	7.17	12.50	11.45	1.044	31.6
11	22	"	7	7.19	14.08	12.95	1.128	34.2
12	30	"	6	7.19	14.22	13.08	1.139	34.5
13	45	"	8	7.20	14.79	13.63	1.160	35.2
14	60	"	11	7.20	15.62	14.39	1.227	37.2
15	75	"	6	7.22	15.75	14.57	1.183	35.9
16	90	"	10	7.23	16.34	15.14	1.202	36.4
17	2	hrs.	8	7.29	16.38	15.32	1.059	32.1
18	2.5	"	7	7.30	16.90	15.83	1.070	32.4
19	3	"	8	7.30	17.27	16.18	1.092	33.1
20	4	"	7	7.40	18.71	17.76	0.954	28.9
21	5	"	8	7.41	18.09	17.19	0.902	27.4
22	6	"	8	7.41	17.28	16.42	0.861	26.1
23	7.3	"	10		17.13			
24	8	"	8		17.10			
25	9	"	8		16.53			
26	9.6	"	10		16.73			

our work shows that at the end of 7 minutes the blood is already well on the way back to normal. Consequently Hamburger missed the maximum effect. Since so many samples were taken, only the pH and carbon dioxide content of the serum were deter-

mined.   An example of one such experiment is given in Table I and Fig. 1.

There was a rapid fall in the pH, the bicarbonate, and carbon dioxide tension which reached its maximum during or at the close of the injection followed by a gradual return toward normal. Reference should be made to Table I for the rapid changes during the injection as not all the points can be shown in the figure.   In
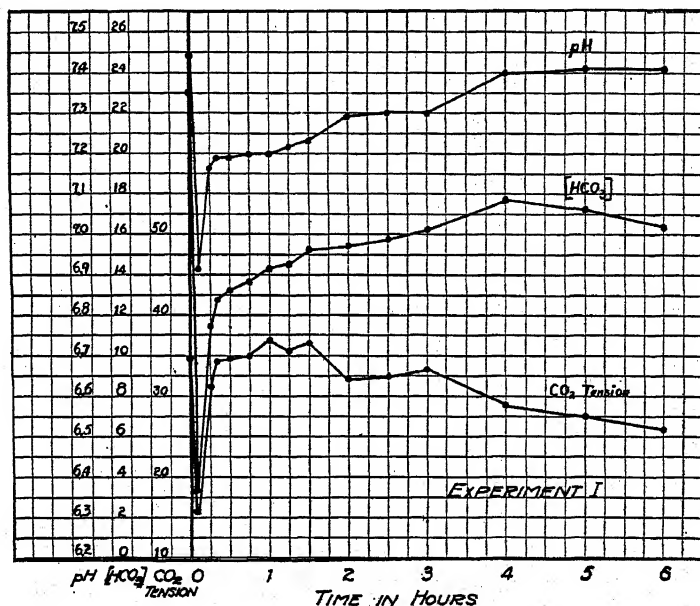
Fig. 1. The change with time in the pH, bicarbonate, and carbon dioxide tension of the blood following the injection of 32 cc. of N hydrochloric acid in Experiment I.   [HCO_3] is expressed in mM per kilo of water, CO_2 tension in mm. of mercury.

the table the zero of time is taken as the beginning of the injection. A time listed as minus is the number of minutes before the injection that the blood was drawn for analysis.   All samples with a time of less than 3 minutes were taken during the injection. Further points of interest are: (1) the extent of the fall, with a pH value of 6.90, bicarbonate below 4 mM per liter, and carbon dioxide tensions below 20 mm.   (2) In some cases it was found that the carbon dioxide tension shows a primary rise during the

TABLE II
Experiment II

Date, May 5, 1927; dog weight, 15.2 kilos; anesthetic, 3.8 gm. barbital; injection, 50 cc. N hydrochloric acid; death, 3¼ hours after injection.

Sample No.	Time	Amount of blood		H₂O		pH		Total [CO₂]	[BHCO₃]	[H₂CO₃]	CO₂ tension	$\frac{[HCO_3]_c}{[HCO_3]_s}$
				Colorimetric	Electrometric							
		cc.		*gm. per cc.*	*gm. per gm.*			*mM per kg. H₂O*	*mM per kg. H₂O*	*mM per kg. H₂O*	*mm. Hg*	
1	−4 min.	20–25	Serum	0.947		7.39	7.32	21.73	20.41	1.317	39.9	1.036
			Cells		0.671			22.45	21.18	1.268	39.9	
2	+3 "	20–25	Serum	0.947		6.20*	6.12	1.12	0.55	0.566	17.2	8.89
			Cells		0.692			5.44	4.89	0.546	17.2	
3	4 "	20–25	Serum	0.943		6.84	6.51	2.52	1.78	0.742	22.5	2.85
			Cells		0.715			5.80	5.08	0.716	22.5	
4	7 "	20–25	Serum	0.949		6.97	6.68	5.28	4.10	1.156	35.1	1.43
			Cells		0.684			7.00	5.88	1.116	35.1	
5	17 "	20–25	Serum	0.951		7.04	6.79	5.94	4.87	1.066	32.3	2.20
			Cells		0.671			11.76	10.73	1.027	32.3	
6	3.25 hrs.	20–25	Serum	0.947		7.28	7.21	13.05	12.05	1.002	30.4	1.32
			Cells		0.673			16.93	15.96	0.966		

* Brom-cresol purple was used as indicator.

injection. This is not a constant result since it occurred in but two of the eight experiments. (3) There is a rapid return toward normal within the first 15 minutes followed by a gradual recovery over a period of hours.

2. Effect of Injection of Acid on Ionic Equilibrium between Cells and Serum of Blood

In the next series of nine experiments, four representative examples of which are included as Experiments II to V (Tables II

TABLE III

Experiment III

Date, May 11, 1927; dog weight, 20 kilos; anesthetic, 5 gm. barbital; injection, 66 cc. N hydrochloric acid; death, 45 minutes after injection.

Sample No.	Time	Amount of blood		H₂O		pH		Total [CO₂]	[BHCO₃]	[H₂CO₃]	CO₂ tension	$\frac{[HCO_3]_c}{[HCO_3]_s}$
						Colorimetric	Electrometric					
	min.	*cc.*		*gm. per cc.*	*gm. per gm.*			*mM per kg. H₂O*	*mM per kg. H₂O*	*mM per kg. H₂O*	*mm. Hg*	
1	−2	20–25	Serum	0.932		7.31	7.30	23.76	22.26	1.504	45.6	0.865
			Cells		0.661			20.71	19.26	1.450	45.6	
2	+3	20–25	Serum	0.930		6.1*	6.14	1.38	0.70	0.682	20.7	2.90
			Cells		0.697			2.69	2.03	0.658	20.7	
3	4	20–25	Serum	0.931		6.5*	6.56	5.83	4.25	1.579	47.9	1.21
			Cells		0.680			6.66	5.14	1.524	47.9	
4	7	20–25	Serum	0.932		6.7*	6.78	7.75	6.33	1.417	43.0	1.45
			Cells		0.675			10.55	9.18	1.367	43.0	
5	18	20–25	Serum	0.932		6.7*	6.82	9.71	8.06	1.647	50.0	1.39
			Cells		0.668			12.79	11.20	1.587	50.0	

* Brom-cresol purple was used as indicator.

to V), it was desired to determine the equilibrium between the cells and serum for a few points on the acid-base path. For this purpose carbon dioxide, chloride, and water determinations were made separately on the cells and the serum. The pH of the serum

TABLE IV

Experiment IV

Date, May 19, 1927; dog weight, 15.7 kilos; anesthetic, 3.7 gm. barbital; injection, 38 cc. N hydrochloric acid; death, 5 hours after injection.

Sample No.	Time	Amount of blood		Index of refraction	H_2O		pH, electrometric	Total $[CO_2]$	$[BHCO_3]$	$[H_2CO_3]$	CO_2 tension	$[Cl]$	$\dfrac{[HCO_3]_c}{[HCO_3]_s}$	$\dfrac{[Cl]_c}{[Cl]_s}$	$\dfrac{r_{Cl}}{r_{HCO_3}}$
		cc.			gm. per cc.	gm. per gm.		mM per kg. H_2O	mM per kg. H_2O	mM per kg. H_2O	mm. Hg	mM per kg. H_2O			
1	−7 min.	35	Serum	1.3532	0.938		7.36	26.18	24.72	1.456	44.2	100.6	0.854	0.732	0.857
			Cells			0.676		22.53	21.12	1.406	44.2	73.6			
2	+3 "	35	Serum	1.3532	0.939		6.81	5.13	4.24	0.887	26.9	119.2	1.485	0.918	0.618
			Cells			0.687		7.15	6.30	0.853	26.9	109.4			
3	4 "	35	Serum	1.3535	0.939		6.81	5.54	4.58	0.958	29.1	113.7	1.224	0.906	0.704
			Cells			0.682		6.54	5.61	0.926	29.1	102.9			
4	7 "	35	Serum	1.3527	0.942		7.04	9.56	8.51	1.046	31.7	113.0	1.065	0.830	0.779
			Cells			0.675		10.07	9.06	1.005	31.7	93.8			
5	15 "	35	Serum	1.3528	0.942		7.07	8.27	7.42	0.851	25.9	110.9	1.058	0.810	0.765
			Cells			0.672		8.67	7.85	0.824	25.9	89.9			
6	3 hrs.	35	Serum	1.3528	0.941		7.07	13.41	12.08	1.386	42.0	106.6	0.875	0.798	0.912
			Cells			0.670		11.91	10.57	1.336	42.0	85.0			

TABLE V

Experiment V

Date, June 15, 1927; dog weight, 22.5 kilos; anesthetic, 5.6 gm. barbital; injection, 50 cc. N hydrochloric acid; death, hours after injection.

Sample No.	Time	Amount of blood		Index of refraction	H_2O		pH, electrometric	Total $[CO_2]$	$[BHCO_3]$	$[H_2CO_3]$	CO_2 tension	$[Cl]$	$\dfrac{[HCO_3]_c}{[HCO_3]_s}$	$\dfrac{[Cl]_c}{[Cl]_s}$	$\dfrac{r_{Cl}}{r_{HCO_3}}$
		cc.			gm. per cc.	gm. per gm.		mM per kg. H_2O	mM per kg. H_2O	mM per kg. H_2O	mm. Hg	mM per kg. H_2O			
1	−2 min.	40	Serum	1.3485	0.946		7.26	27.53	25.63	1.900	57.6	110.1	0.902	0.730	0.809
			Cells			0.655		24.96	23.13	1.832	57.6	80.3			
2	+3 "	40	Serum	1.3491	0.943		6.84	10.47	8.76	1.708	51.8	123.8	1.124	0.902	0.802
			Cells			0.678		11.50	9.85	1.648	51.8	111.6			
3	4 "	40	Serum	1.3489	0.944		7.18	11.34	10.41	0.928	28.2	131.5	1.035	0.775	0.748
			Cells			0.667		11.68	10.78	0.897	28.2	101.9			
4	7 "	40	Serum	1.3488	0.945		7.19	15.97	14.69	1.279	38.8	120.2	0.998	0.787	0.789
			Cells			0.667		15.90	14.67	1.234	38.8	94.6			
5	15 "	40	Serum	1.3485	0.944		7.19	17.31	15.92	1.386	42.0	123.8	0.955	0.752	0.787
			Cells			0.665		16.55	15.21	1.336	42.0	93.1			
6	4 hrs.	40	Serum	1.3485	0.945		7.18	22.67	20.81	1.855	56.3	115.5	0.990	0.768	0.776
			Cells			0.667		22.40	20.61	1.790	56.3	88.8			

was also determined. Because of the large number of analyses necessary on each sample, only a few samples could be drawn in each experiment. It was shown by Van Slyke, Wu, and McLean (4) that the distribution of the diffusible ions in the serum and cells is related to the non-diffusible ions by the formula:

$$r = \frac{[H^+]_s}{[H^+]_c} = \frac{[Cl^-]_c}{[Cl^-]_s} = \frac{[HCO_3^-]_c}{[HCO_3^-]_s} = 1 - \frac{[BP]_c + [Hb]_c - [BP]_s}{2\,([B]_s - [BP]_s)}$$

where

$$[BP]_c = 3.35\,[Hb]_s\,(pH_c - 6.74) + [O_2]\,(pH_c - 1.18)$$

$$[BP]_s = 0.068\,[P]_s\,(pH_s - 4.80)^*$$

It was possible therefore to calculate a relation between pH and the distribution of the diffusible ions, r. They tested this formula experimentally on horse blood equilibrated at different carbon dioxide tensions at varying pH values, and found good agreement between the calculated and determined ratios. It was subsequently found in Van Slyke's laboratory that the stoichiometric bicarbonate and chloride ratios, although not identical, vary with the pH in the manner predicted by the Van Slyke, Wu, and McLean equation. This has been attributed to a difference in the activity coefficient of the two ions within the cells. With this exception, experiments *in vitro* have generally been quite in agreement with the theory. There remained, however, the testing of the hypothesis on blood *in vivo*.

Experiments II and III are interesting in that they include extremely low pH values. In Experiment II the pH at the end of injection (the 3 minute sample) was 6.12 electrometrically and was checked colorimetrically as 6.20. This dog died 3 hours and 15

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* The equations quoted here for BP_c and BP_s hold for dialyzed horse cells and serum, whereas in our experiments dog blood has been used exclusively. Data on the base bound by dog hemoglobin are available but the titration curves of dialyzed dog cells and serum have not as yet been determined. Until this has been done, it seems unprofitable to attempt to modify the Van Slyke, Wu, and McLean equations so that they hold for dog blood. This is particularly true since the differences involved are outside the limits of error of our experiments.

minutes after the injection. In Experiment III, the pH at the end of injection was 6.14 electrometrically and was checked as 6.10 colorimetrically. This dog died 45 minutes later.

In Fig. 2 the $[Cl]_s$, $[Cl]_c$, $[BHCO_3]_s$, and $[BHCO_3]_c$ for Experiment IV are plotted against time. This figure shows the following points of interest: (1) a greater increase in chloride concentration in the cells than in the serum; (2) a more rapid subsequent fall in
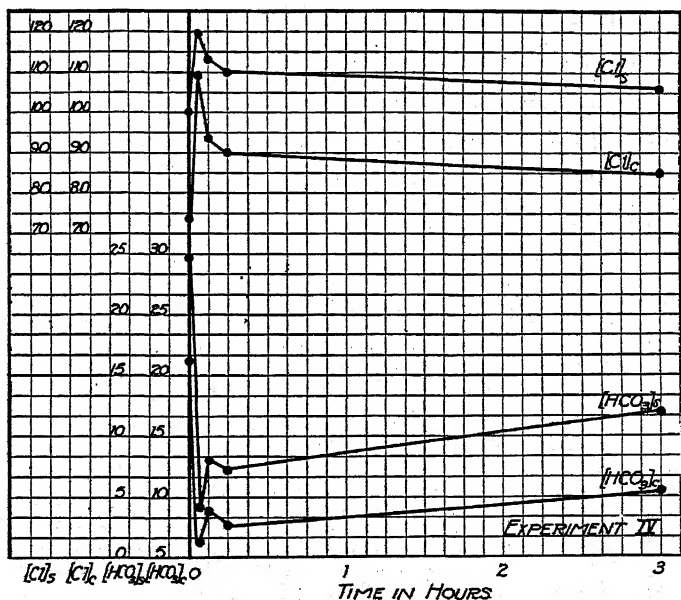
FIG. 2. The change with time in the chloride and bicarbonate concentration of the serum and cells. $[Cl]_s$, $[Cl]_c$, $[HCO_3]_s$, and $[HCO_3]_c$ are expressed in mM per kilo of water.

chloride concentration in the cells than in the serum; (3) a greater decrease in bicarbonate concentration in the serum than in the cells; (4) a more rapid subsequent rise in bicarbonate concentration in the serum than in the cells.

In Fig. 3 the chloride ratios plotted against pH are given for three of the *in vivo* experiments and for two *in vitro* experiments. There is variation in the level and slope of the curves for the individual dogs, but in general the experimental points follow the

curves plotted from the equation. In the case of the corresponding bicarbonate ratios shown in Fig. 4, there is fair consistency except in the case of one point from Experiment IV.

The theory calls for a distribution ratio of approximately unity at the isoelectric point of hemoglobin. This is realized in the case of the chlorides, but in the case of the bicarbonate the ratio exceeds unity long before the isoelectric point is reached. This may indicate that the calculated bicarbonate values for cells are much
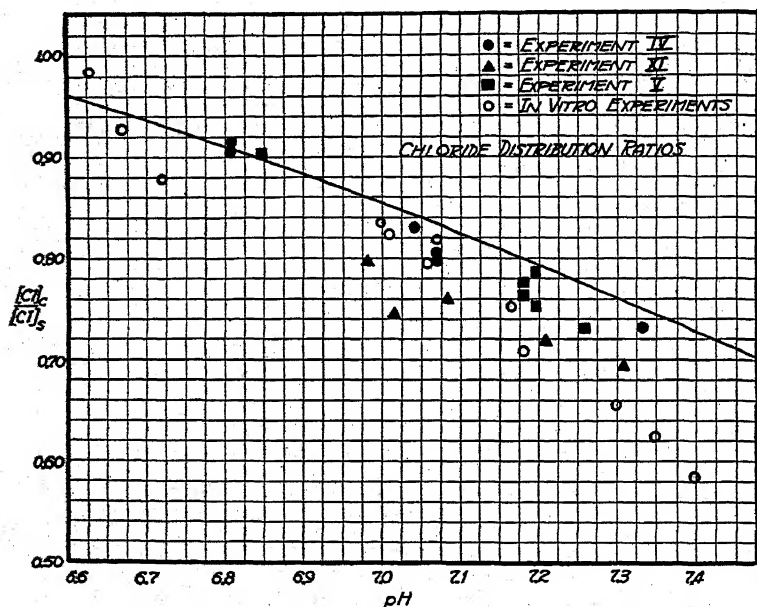
FIG. 3. The change in the chloride distribution ratios with pH. The ratios of two *in vitro* experiments and three *in vivo* experiments are included.

too high. Whether this is due to there being an appreciable amount of undissociated hemoglobin bicarbonate or to activity changes is uncertain. The Donnan distribution law predicts a higher concentration of diffusible ions in cells than in serum when the non-diffusible ion, in this case hemoglobin acting as a base, is a cation. In other experiments not shown in Fig. 4 where the pH was much below the isoelectric point of hemoglobin, there were found bicarbonate ratios much greater than unity.

3. Effect of Injection of Acid on the Urine

In only one experiment (Experiment VI, Table VI) was a urine analysis made. In all, however, it was noticed that urinary excretion was slight, never occurring spontaneously, and at death the bladder was practically empty. In Experiment VI, only about 8 cc. of urine were obtained by catheterization. This was only a little more acid than the control specimens and contained a very
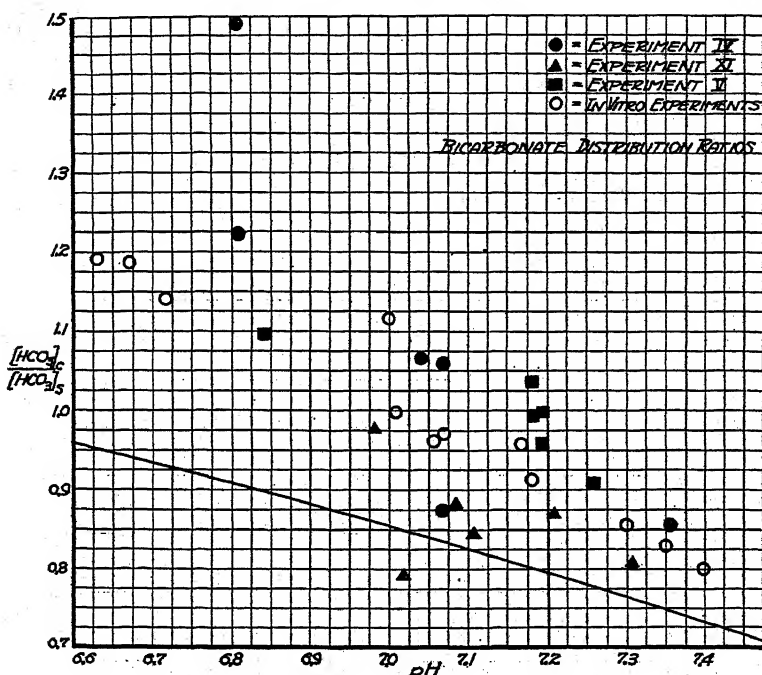
Fig. 4. The change in the bicarbonate distribution ratios with pH. The ratios of two *in vitro* experiments and three *in vivo* experiments are included.

low concentration of chloride. The secretion of urine per minute and of chloride per minute were both diminished and the total chloride excretion after the injection was only equal to 0.08 per cent of the amount injected.

4. Effect of Injection of Acid on the Respiration and Blood Pressure

Blood pressure tracings were made in Experiment VI and one other experiment not included in the tables. A fall of from 20 to

TABLE VI
Experiment VI

Date, December 2, 1928; dog weight, 16.5 kilos; anesthetic, 4.13 gm. barbital; injection, 40 cc. N hydrochloric acid; death, 23 hours after injection.

Sample No.	Time	Amount of blood (cc.)		Index of refraction	H₂O (gm. per cc.)	H₂O (gm. per gm.)	pH Colorimetric	pH Electrometric	Total [CO₂] (mM per kg. H₂O)	[BHCO₃] (mM per kg. H₂O)	[H₂CO₃] (mM per kg. H₂O)	CO₂ tension (mm. Hg)	[Cl] (mM per kg. H₂O)	$\frac{[HCO_3]_c}{[HCO_3]_s}$	$\frac{[Cl]_c}{[Cl]_s}$	$\frac{r_{Cl}}{r_{HCO_3}}$
1	−30 min.	40	Serum	1.3540	0.930	0.668	7.39	7.38	19.51	18.47	1.038	31.2	106.4	0.774	0.782	1.010
			Cells						15.29	14.30	0.992	31.2	83.2			
2	+3 "	40	Serum	1.3538	0.929	0.677	6.92	6.81	6.08	5.03	1.050	31.9	114.1	1.067		
			Cells						6.38	5.37	1.014	31.9				
3	4 "	40	Serum	1.3537	0.929	0.677	7.14	6.99	7.00	6.14	0.859	26.0	116.2	0.875	0.892	1.019
			Cells						6.20	5.37	0.827	26.0	103.7			
4	7 "	40	Serum	1.3531	0.932	0.676	7.24	7.15	6.92	6.32	0.603	18.3	118.0	0.940		
			Cells						6.52	5.94	0.582	18.3				
5	19 "	40	Serum	1.3534	0.930	0.672	7.24	7.16	6.64	6.07	0.566	17.2	126.9	0.919	0.778	0.847
			Cells						6.13	5.58	0.547	17.2	98.7			
6	5 hrs.	60	Serum	1.3534	0.931	0.670	7.28	7.25	12.51	11.63	0.883	26.8	120.3	0.886	0.756	0.854
			Cells						11.16	10.31	0.852	26.8	91.0			

(Hydrochloric acid injection) urinalysis

Sample No.	Time after injection	Amount of urine	pH, colorimetric	[Cl]	Total [Cl]	Urine	[Cl]
		cc.		mM per kg. H₂O	mM	cc. per min.	mM per min.
1	Control	4.0	6.35			0.09	
2	"	2.5	6.12	35	0.14	0.06	0.003
3	" [to +3	1.2	5.98	38	0.09	0.03	0.003
4	43		5.92			0.01	
5	330	3.0	6.15	63	0.25		0.001

40 mm. of mercury was encountered in these experiments with a return to normal in 1 or 2 hours. The fall occurred gradually, not beginning till near the end of the injection and not reaching the minimum till about 8 minutes later.

In almost all of the experiments continuous respiratory tracings were taken. The results were quite uniform. A typical tracing is shown in Fig. 5. About 20 seconds after the start of the injection a period of hyperpnea began. The onset of the hyperpnea was quite sudden. Usually there was an increase in both the rate and amplitude of respiration, but in a few cases the amplitude alone was increased. The suddenness of the onset seems to indicate some sudden action on the respiratory regulatory system. This is most probably coincident with the arrival of the acidified blood in the medulla, but could possibly be from reflexes in the lungs or peripheral nerves. In the various experiments this interval, which we took to represent the circulation time from the femoral vein to the right heart, through the pulmonary circuit to the left heart and from there to the medulla, was 19, 12, 32, 20, 18, 10, 22, 16, and 20 seconds, averaging 17 seconds (the aberrant 32 was not included in the average). According to Vierordt the time for complete circulation in the dog is 16.3 seconds. If 3 seconds is added to our value, since it does not include the complete circuit, a value of 20 seconds would be obtained for the complete circulation time.

The next observation on the breathing was that the hyperpnea continued throughout the injection and for about half a minute to a minute afterwards. Then followed a period of breathing in which the diminished rate and amplitude were marked features. Then there was a gradual return to normal, with a late gradual weakening and slowing of the respiration as death approaches.

The relation of the acid-base condition of the blood to respiratory activity has been much discussed, but divergent conclusions have been reached. Haldane, from a study of the relation of alveolar carbon dioxide to respiratory activity, concluded that the carbon dioxide tension of the alveolar air, and consequently the carbon dioxide tension of arterial blood, is the respiratory hormone. Winterstein, Hasselbalch, and others support the view that the hydrogen ion concentration of the arterial blood is the normal respiratory hormone. Lately Gesell has taken the view that the
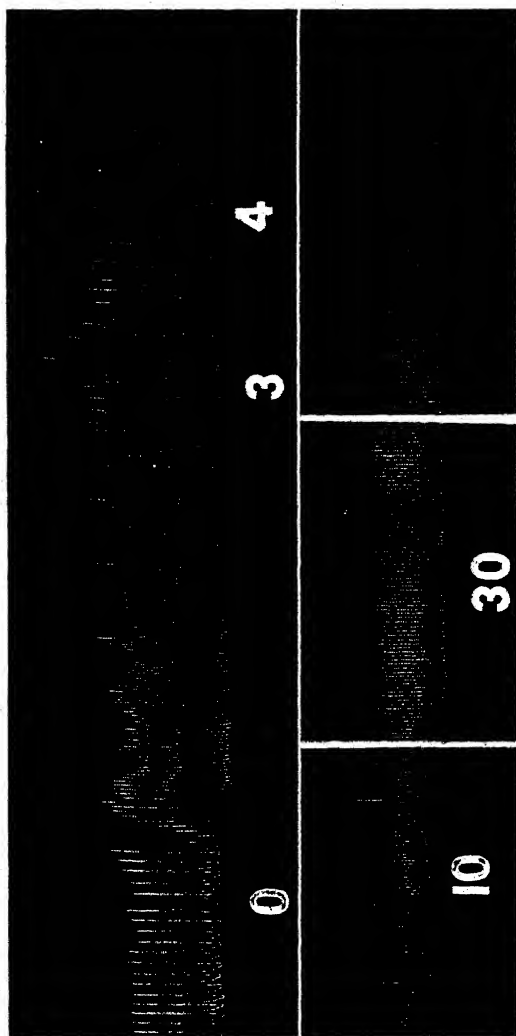
Fig. 5. Pneumographic respiratory tracing in Experiment II. The numbers denote the number of minutes after the injection of 50 cc. of N hydrochloric acid. The injection was started at 0 and ended at 3. The section of the tracing in the lower right-hand corner shows the death of the animal 3 hours and 15 minutes after the injection.

hydrogen ion concentration within the respiratory center is the ultimate stimulus to respiratory activity and that the constituents of the blood are important only in so far as they modify the reaction of the cells in the medulla. By means of simultaneous measurements of the reaction of the cerebrospinal fluid and the blood, Gesell found instances in which there was increased alkalinity of the blood, but increased acidity of the spinal fluid.
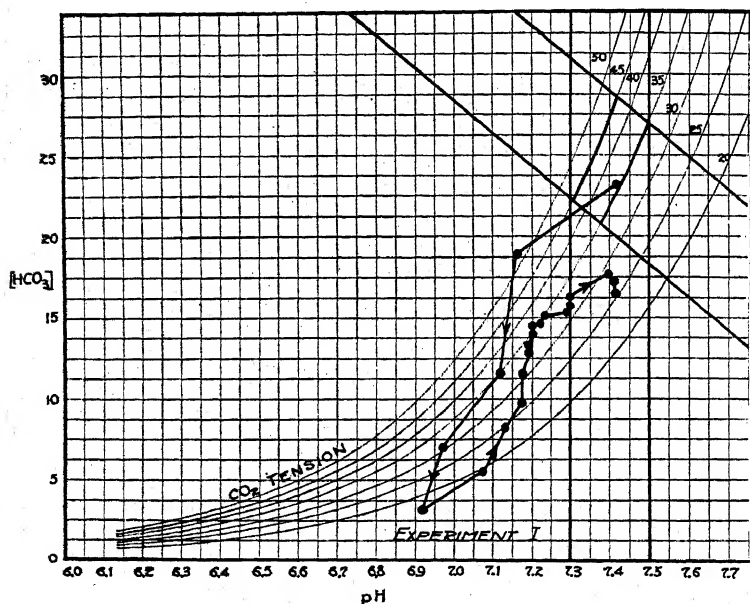
Fig. 6. The path followed by the acid-base condition of the blood during and following the injection of 32 cc. of N hydrochloric acid in Experiment I. The arrows indicate the course followed by the blood. [HCO₃] is expressed in mM per kilo of water.

Associated with this was increased respiratory activity. From a consideration of the path taken by the blood in a respiratory cycle when considered from the standpoint of the acid-base balance of blood, Murray and Hastings concluded that respiratory activity is correlated with changes in the carbon dioxide tension of the blood.

The relation that our experiments bear to these hypotheses

may be shown by reference to the acid-base chart of the form first suggested by Van Slyke. Two experiments are shown in Figs. 6 and 7. These charts have the bicarbonate plotted as ordinates, the pH as abscissæ, and have iso-carbon dioxide tension curves running diagonally across the chart. The arterial blood of normal individuals will fall between the area bounded by pH 7.3 and 7.5,
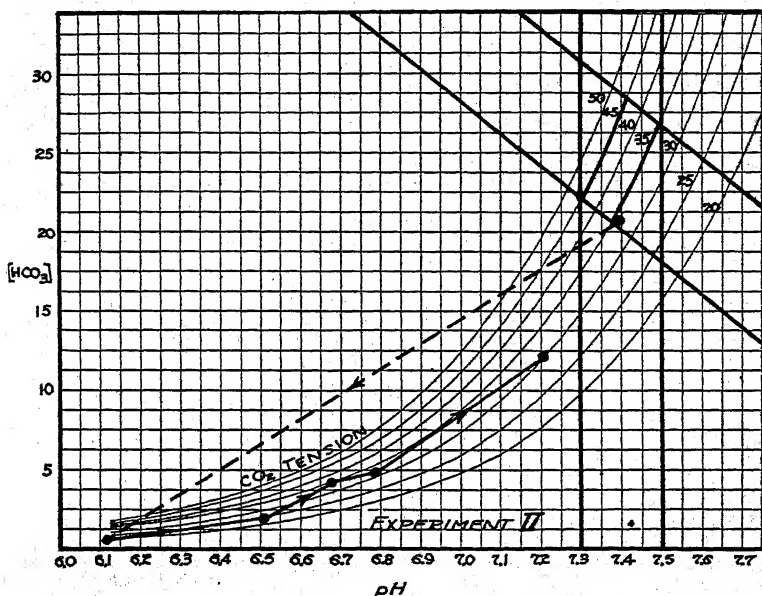
Fig. 7. The path followed by the acid-base condition of the blood during and following the injection of 50 cc. of N hydrochloric acid in Experiment II. The arrows indicate the course followed by the blood. [HCO₃] is expressed in mM per kilo of water.

carbon dioxide tension 35 and 45 mm. of mercury, and the carbon dioxide absorption curves as indicated.

Deviation from the normal condition might be in any of several directions. Suppose that the purpose of respiration is to keep the pH constant. Then any diminution in the bicarbonate as produced by hydrochloric acid would be followed by respiratory activity of such an extent that the fall in carbon dioxide tension would be proportional to the fall in bicarbonate and the change

would be in the direction of a vertical line on the chart. If, however, the purpose of respiration were to maintain a constant carbon dioxide tension, then one would expect the change pro-
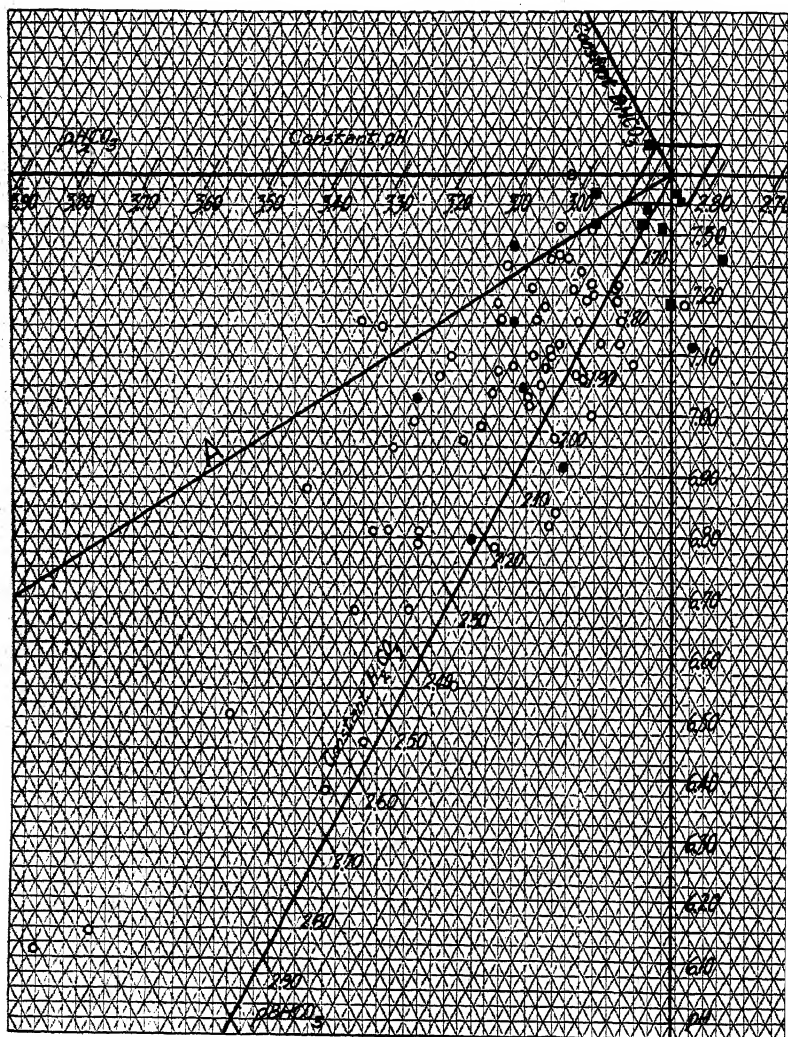
Fig. 8. Acid-base data on the blood of dogs during experimental acidosis plotted logarithmically on triaxial coordinates.

duced by hydrochloric acid to follow along an iso-carbon dioxide tension line.

The results shown in Fig. 6 represent a typical finding. During the injection, the carbon dioxide tension rises slightly above normal. At the end of the injection it is below normal but soon returns toward normal. In general the paths of injection and recovery follow the iso-carbon dioxide tension lines much more closely than they do the iso-pH lines.

Although plotting acid-base data on rectangular coordinates is the usual method, certain points stand out more clearly if they are plotted on triaxial coordinate paper. The coordinates on such paper make 60° angles with one another. By plotting acid-base data logarithmically on such paper the three variables, pH, bicarbonate, and carbon dioxide tension all bear the same graphical relation one with the other. This avoids the difficulty of interpreting acid-base paths as plotted in the usual manner with bicarbonate as ordinates and pH as abscissæ. Iso-carbon dioxide tension lines on rectangular coordinates are curves and run diagonally across the chart. Furthermore at low pH, the carbon dioxide tension lines approach each other so that it is difficult to determine whether there has been a significant deviation from the initial carbon dioxide tension value. When plotted on triaxial coordinate paper these difficulties are obviated. All of the experimental points, 62 in all, determined on the blood of dogs in which experimental acidosis has been produced, have been plotted on such a chart, Fig. 8. It is obvious at once from an inspection of this chart that there is no tendency for the acid-base balance of the blood to follow a constant pH line under the conditions of our experiments. Furthermore, by far the largest majority of the points fall well to the left of the constant carbon dioxide tension line which corresponds on the chart to 40 mm. Although it has been stated that the acid-base balance of the blood gives an average between carbon dioxide tension and pH, it is also apparent from Fig. 8 that this statement is not strictly correct. The line marked A represents the direction that the acid-base balance of the blood would take if a perfect compromise were effected. Practically all of our points lie to the right of this line. It may be concluded from our experiments that the direction taken by the acid-base balance of the blood when it is displaced markedly from

its normal condition by fixed acid, is in the direction which is approximately three times as far removed from a constant pH line as from a constant carbon dioxide tension line.

Further evidence of the lack of correspondence between arterial blood pH and respiratory activity is the fact that although the pH remains below normal for about 3 hours after the injection, the respiration is normal during this period. At no time has hyperpnea as measured by the pneumograph been encountered following the period of injection. Professor Carlson raised the question of whether the lack of response of the respiratory center to the low pH might be due to paralysis of the center from the action of the strong acid. To test this point, we tested the activity of the center to minimal stimuli of the faradic current applied to the central ends of the sciatic and vagus nerves, and found that the activity of the center is the same before and after the injection. This result was duplicated in two experiments, Experiment VI and one other experiment not included in the tables. Further evidence that the activity of the center is not depressed significantly is that after the injection the carbon dioxide tension of the blood is kept at or below the normal value. It is to be concluded from these observations that our experiments indicate that the pH of itself cannot be the respiratory hormone, nor can it be concluded that the carbon dioxide tension of the blood alone is the hormone. Perhaps the pH and carbon dioxide tension are only important in that their alteration is only a symptom of a displacement of the system of which they are a dependent part. From a teleological view-point, to say that the respiratory center would arbitrarily regulate one of these factors and leave the other to accomplish its own *restitutio ad integram* seems illogical. This view would be in harmony with the work of Gesell, for to affect the condition in the respiratory center all the blood factors would enter in: pH, bicarbonate, carbon dioxide tension, etc., one perhaps more important than another, but all having a share in the integration of a general alteration of the acid-base equilibrium.

5. Control Experiments

Experiments VII and VIII (Tables VII and VIII) serve as controls for the injection experiments. The dogs were submitted to exactly the same régime, even to the extent of tying off the

TABLE VII

Experiment VII, Control

Date, June 17, 1927; dog weight, 10.9 kilos; anesthetic, 2.7 gm. barbital; injections, none.

Sample No.	Time	Amount of blood		Index of refraction	H_2O	pH Colorimetric	pH Electrometric	Total $[CO_2]$	$[BHCO_3]$	$[H_2CO_3]$	CO_2 tension	$[Cl]$	$\dfrac{[HCO_3]_c}{[HCO_3]_s}$	$\dfrac{[Cl]_c}{[Cl]_s}$	$\dfrac{r_{Cl}}{r_{HCO_3}}$
		cc.			*gm. per cc.* / *gm. per gm.*			*mM per kg. H_2O*	*mM per kg. H_2O*	*mM per kg. H_2O*	*mm. Hg*	*mM per kg. H_2O*			
1	−7 min.	40	Serum	1.3503	0.944	7.35	7.30	29.64	27.76	1.879	57.0	110.5	0.836	0.706	0.844
			Cells		0.657			25.01	23.20	1.812	57.0	78.0			
2	+3 "	40	Serum			7.36	7.33	28.09	27.42	1.666	50.6				
			Cells		0.657										
3	4 "	40	Serum			7.38	7.24	28.12	26.10	2.024	61.4				
			Cells												
4	7 "	40	Serum			7.38	7.30	27.97	26.20	1.770	53.7				
			Cells												
5	15 "	40	Serum	1.3499	0.944	7.38	7.30	27.80	26.04	1.760	53.4	114.2	0.821	0.673	0.820
			Cells		0.659			23.06	21.36	1.698	53.4	76.9			
6	3.5 hrs.	40	Serum	1.3499	0.945	7.34	7.21	23.66	21.84	1.817	55.1	111.6	0.827	0.692	0.837
			Cells		0.657			19.82	18.07	1.752	55.1	77.2			
7	7.2 "	40	Serum	1.3496	0.947	7.22	7.17	24.15	22.13	2.020	61.0	114.2	0.813	0.680	0.837
			Cells		0.655			19.92	17.98	1.940	61.0	77.6			

TABLE VIII
Experiment VIII, Control

Date, June 23, 1928; dog weight, 18 kilos; anesthetic, 4.5 gm. barbital; injection, none; death, 8 hours.

Sample No.	Time	Amount of blood		Index of refraction	H₂O	H₂O	pH, colorimetric	Total [CO₂]	[BHCO₃]	[H₂CO₃]	CO₂ tension	[Cl]	$\frac{[HCO_3]c}{[HCO_3]s}$	$\frac{[Cl]c}{[Cl]s}$	$\frac{rCl}{rHCO_3}$
		cc.			gm. per cc.	gm. per gm.		mm per kg. H₂O	mm per kg. H₂O	mm per kg. H₂O	mm. Hg	mm per kg. H₂O			
1	−2 min.	40	Serum	1.3536	0.926		7.34	21.73	20.47	1.262	38.3	122.5	0.847	0.692	0.818
			Cells			0.646		18.57	17.35	1.217	38.3	84.8			
2	+3 "	40	Serum				7.35	20.53	19.36	1.166	35.4				
			Cells							1.125	35.4				
3	4 "	40	Serum				7.37	20.40	19.29	1.110	33.7				
			Cells							1.071	33.7				
4	7 "	40	Serum				7.36	21.30	20.12	1.184	35.9				
			Cells							1.142	35.9				
5	15 "	40	Serum	1.3522	0.929		7.37	20.31	19.20	1.105	33.5	125.2	0.823		
			Cells			0.644		16.87	15.80	1.065	33.5				
6	1.7 hrs.	40	Serum	1.3523	0.928		7.36	20.30	19.17	1.128	34.2	125.2	0.781	0.675	0.865
			Cells			0.644		16.05	14.96	1.086	34.2	84.5			
7	5.05 "	300	Serum	1.3521	0.930		7.45	18.50	17.65	0.846	25.9	127.8	0.756	0.649	0.859
			Cells			0.649		14.17	13.35	0.824	25.9	82.9			

TABLE IX

Experiment IX, in Vitro Experiment

Date, October 10, 1927. The arterial defibrinated blood was saturated at various carbon dioxide tensions and equilibrated at 38°. Samples 4, 5, and 6 were acidified in the proportion of 2 cc. of N hydrochloric acid to 200 cc. of blood before the saturation and equilibration.

Sample No.		H₂O		pH		Total [CO₂]	[BHCO₃]	[H₂CO₃]	CO₂ tension	[Cl]	$\frac{[HCO_3]_c}{[HCO_3]_s}$	$\frac{[Cl]_c}{[Cl]_s}$	$\frac{r_{Cl}}{r_{HCO_3}}$
				Colorimetric	Electrometric								
		gm. per cc.	*gm. per gm.*			*mM per kg. H₂O*	*mM per kg. H₂O*	*mM per kg. H₂O*	*mm. Hg*	*mM per kg. H₂O*			
1	Serum	0.941		7.43	7.40	19.26	18.28	0.982	29.8	117.8	0.800	0.584	0.730
	Cells		0.646			15.56	14.61	0.948	29.8	68.8			
2	Serum	0.939		7.38	7.35	21.80	20.58	1.224	36.8	114.9	0.827	0.623	0.753
	Cells		0.653			18.20	17.03	1.173	36.8	71.6			
3	Serum	0.940		7.31	7.30	24.36	22.82	1.543	46.8	108.9	0.856	0.657	0.767
	Cells		0.656			21.01	19.53	1.484	46.8	71.5			
4	Serum	0.940		7.26	7.18	10.59	9.72	0.867	26.3	124.4	0.912	0.709	0.777
	Cells		0.666			9.70	8.86	0.836	26.3	88.2			
5	Serum	0.932		7.24	7.17	13.33	12.22	1.113	33.8	123.7	0.959	0.756	0.788
	Cells		0.668			12.80	11.72	1.075	33.8	93.5			
6	Serum	0.936		7.10	7.06	15.53	13.90	1.633	49.5	131.7	0.964	0.794	0.823
	Cells		0.672			14.97	13.40	1.574	49.5	104.6			

TABLE X
Experiment X, in Vitro Experiment

Date, October 26, 1927. The arterial defibrinated blood was saturated at various carbon dioxide tensions and equilibrated at 38°. Samples 4, 5, and 6 were acidified in the proportion of 3 cc. of N hydrochloric acid to 200 cc. of blood before the saturation and equilibration.

Sample No.		H_2O		pH		Total $[CO_2]$	$[BHCO_3]$	$[H_2CO_3]$	CO_2 tension	$[Cl]$	$\dfrac{[HCO_3]_c}{[HCO_3]_s}$	$\dfrac{[Cl]_c}{[Cl]_s}$	$\dfrac{r_{Cl}}{r_{HCO_3}}$
		gm. per cc.	gm. per gm.	Colorimetric	Electrometric	m.m. per kg. H_2O	m.m. per kg. H_2O	m.m. per kg. H_2O	mm. Hg	m.m. per kg. H_2O			
1	Serum	0.931		7.09	7.07	7.65	6.86	0.788	23.9	115.8	0.971	0.819	0.843
	Cells		0.663			7.42	6.66	0.760	23.9	94.9			
2	Serum	0.926		7.01	7.00	11.56	10.19	1.374	41.7	117.5	1.185	0.836	0.706
	Cells		0.664			13.41	12.08	1.326	41.7	98.3			
3	Serum	0.929		7.06	7.01	12.66	11.19	1.474	44.7	112.8	0.999	0.822	0.823
	Cells		0.665			12.60	11.18	1.421	44.7	92.8			
4	Serum	0.930		6.75	6.72	2.73	2.17	0.558	16.9	129.8	1.147	0.880	0.767
	Cells		0.676			3.03	2.49	0.537	16.9	114.3			
5	Serum	0.931		6.69	6.67	4.97	3.86	1.112	33.7	126.6	1.189	0.929	0.781
	Cells		0.677			5.66	4.59	1.072	33.7	117.7			
6	Serum	0.933		6.70	6.63	8.02	6.09	1.926	58.4	122.0	1.195	0.981	0.821
	Cells		0.679			9.14	7.28	1.858	58.4	119.7			

femoral vein. Anesthesia, artery cannulation, sample withdrawals, and analyses were performed identically the same as in the injection experiments. The important observations noted in the controls are as follows: (1) While in the injection experiments the refractive indexes tended to rise slightly, in the control experiments they fell slightly. (2) While in the injection experiments the serum water fell markedly, in the controls the serum water rose slightly and the cell water remained constant. (3) In the controls the pH in one experiment, and the serum and cell bicarbonate in both, showed a late drop. This may account for the late drop noted in the acidosis experiments after these values were practically normal. (4) The bicarbonate and chloride ratios showed a slight drop whereas in the acidosis experiments they showed a marked rise.

6. In Vitro Treatment of Blood with Hydrochloric Acid

The in vitro experiments, Nos. IX and X (Tables IX and X), were performed as follows: A large sample of blood from a single dog was divided into two parts. The second part was acidified with a small amount of hydrochloric acid. Then samples from each part were saturated at various carbon dioxide tensions. In general, as seen from the tables and from Figs. 3 and 4, the reaction of blood to fixed acid is quite similar in vivo and in vitro. A point of similarity rests in the observation that in Experiment X bicarbonate ratios of greater than unity were encountered when the pH was below 7.0.

CONCLUSIONS

1. The effect of the injection of large amounts of hydrochloric acid consists of an increase in refractive index of the serum, decrease in water content of the serum, increase in water content of the cells, marked lowering of the pH of serum and the bicarbonate of cells and serum, increase in the chloride of cells and serum, and increase in the bicarbonate and chloride ratios between cells and serum.

2. Extremely low values of the pH (6.12 and 6.14 in two of the experiments) were encountered momentarily immediately following the injection of the acid.

3. The experiments have in general verified the hypothesis that

the diffusible ions HCO_3^- and Cl^- are distributed in the blood according to the Donnan distribution law, in spite of the production of a severe experimental acidosis.

4. Since the pH of blood sometimes fell below the isoelectric point of hemoglobin, causing a bicarbonate ratio greater than unity (even allowing for the lowered activity in the cells), the validity of the Van Slyke, Wu, and McLean hypothesis has been further strengthened.

5. The injection caused an anuria, so that very little of the injected chloride is excreted.

6. The injection caused a marked hyperpnea which set in suddenly about 20 seconds after the start of the injection. This is probably due to the arrival of acidified blood in the respiratory center.

7. The fact that for some time after the injection the pH remains very low whereas the respiration soon returns to normal is contradictory to the belief that the pH is the respiratory hormone. This contradiction is further strengthened when it is considered that during this interval the respiratory center remains responsive to a minimal faradic stimulus applied to the central ends of the vagus and sciatic nerves. Moreover, the path of injection and recovery does not at all follow an iso-pH line as it would tend to do if the pH were the respiratory hormone. It seems probable that the pH is not the sole respiratory hormone, but yet it is not convincing that the carbon dioxide tension is the stimulus *sine qua non*. As a logical compromise it is possible that the pH and carbon dioxide tension are only important in that they are factors in a general alteration of the acid-base equilibrium.

8. The physicochemical laws found to be adequate in predicting the distribution of diffusible ions between cells and serum *in vitro* are also adequate to account for their distribution in acute experimental acidosis *in vivo*.

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CAROTENE

III. HYDROGENATION AND OPTICAL PROPERTIES OF CAROTENE AND ITS HYDROGENATED DERIVATIVES

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The empirical formula of carotene, $C_{40}H_{56}$, shows that there are thirteen unsaturated linkages in the molecule. In order to determine whether these unsaturated linkages are double bonds, rings, or combinations of the two, catalytic hydrogenation has been used.

Escher (1) made the first attempt to hydrogenate carotene catalytically but did not succeed. 20 years later Zechmeister and his coworkers ((2) p. 566), employing improved methods, succeeded in adding 11 mols of hydrogen. They reported that this compound, perhydrocarotene, was a paraffin-like solid, melting at 65° ((2) p. 568) and had either no optical rotation or such a slight rotation as to be immeasurable (3). They observed that during hydrogenation the carotene solution became colorless when 9 mols of hydrogen had been absorbed. On comparing the disappearance of color with mols of hydrogen added, they found (4) that carotene and methyl bixin behaved similarly during the addition of the first 9 mols of hydrogen. Since Kuhn (5) had shown previously that methyl bixin contained a system of nine conjugated double bonds the Hungarian workers (4) concluded that carotene also contained the same grouping.

Our attempts to make perhydrocarotene by catalytic hydrogenation using platinum oxide catalyst were unsuccessful, even though partial hydrogenation did occur. The products obtained had physical properties entirely different from perhydrocarotene. This observation indicated that the investigation of a number of intermediate products of hydrogenation might yield valuable information as to the structure of carotene.

As a result of this investigation we have obtained compounds containing 1, 9, and 10 mols of additional hydrogen. Observations on the optical properties of carotene and its hydrogenated derivatives have shown that carotene is optically active and that the optical rotation is greatly affected by progressive hydrogenation. The observations further indicate that complete hydrogenation symmetrizes the molecule, that there is a conjugated system of nine double bonds, and that there may be present in the carotene molecule two bicyclic rings similar to carane or thujane. While the physical measurements point to these possibilities, it will remain for organic methods to establish them.

<div align="center">EXPERIMENTAL</div>

Optical Properties of Carotene

Although Kohl (6) reported a specific rotation of $-30.17°$ for carotene in chloroform solution, Karrer (7) recently reported that it was optically inactive. Since it is important to establish the symmetry or asymmetry of the molecule the optical rotation was redetermined. The results show that carotene is optically active.

Since solutions of carotene were so red that the rotation could not be determined with the ordinary light sources, it was necessary to use a carbon arc.

In chloroform the observed rotations were no larger than the experimental errors, therefore a value for this solvent will not be reported. In carbon bisulfide, however, the magnitudes of the observed rotations were so large as to be incontestable.

In carbon bisulfide the specific rotation was found to be

$$[\alpha]_D^{15} = \frac{-0.161° \times 100}{1 \times 0.2544} = -63° \pm 17 \text{ per cent}$$

The absorption spectrum curve for carotene in cyclohexane solution, plotted in Fig. 1, is taken from a communication of Pummerer, Rebmann, and Reindel (8). In visible light it agrees well with values we obtained for an ethyl alcoholic solution (9).

Reduction of Carotene with Aluminum Amalgam. Dihydrocarotene

A reduction product of carotene, containing 2 additional atoms of hydrogen, was prepared by reducing carotene in ether solution with aluminum amalgam.

0.951 gm. of purified carotene, 250 cc. of ether, and 50 gm. of aluminum amalgam were put into an Erlenmeyer flask and the flask attached to a reflux condenser through a ground glass connection. Vigorous action ensued, and after 10 minutes the solution became bright yellow. As no further change was apparent during the next 3 hours the reaction mixture was filtered free of solids and the filtrate concentrated to less than 50 cc. The concentrate was transferred to a 50 cc. volumetric flask and diluted to the mark with ether.

The optical rotation of the solution was then observed and the weight of the solute found after evaporating the solution to dryness. From these data the specific rotation was calculated.

$$[\alpha]_D^{18} = \frac{+ 1.39° \times 50}{2 \times 0.83} = + 41.9°$$

The residue, from the evaporation of the ether, was further dried for 40 hours at 60° and 10^{-3} mm. mercury pressure. It was an orange, viscous, non-crystallizable substance which was more soluble in the organic solvents tried than carotene. Analyses showed that it was dihydrocarotene.

$C_{40}H_{58}$. Calculated. C 89.14, H 10.86
Found.   " 88.97, " 10.74
                " 89.09, " 10.76

A portion of the residue dissolved in ethyl alcohol also showed dextrorotation.

$$[\alpha]_D^{18} = \frac{+ 0.176° \times 100}{2 \times 0.2316} = + 38.0°$$

By using a Hilger quartz spectrograph (E37) and rotating sector photometer the absorption spectrum of dihydrocarotene in ethanol was obtained. The absorption curve is shown in Fig. 1.

Catalytic Hydrogenation of Carotene

The catalytic hydrogenation of carotene with Adams' catalyst (10) was next undertaken. The degree of hydrogenation was found to vary with the amount of catalyst used. For this reason experiments with both high and low ratios of catalyst to carotene will be described.

When the ratio of catalyst to carotene was low, 1 mol of carotene was found to absorb 9 mols of hydrogen; when the ratio was high, it absorbed 10 mols of hydrogen.

Low Ratio of Catalyst to Carotene. Octadecahydrocarotene

First, experiments in which a low ratio of catalyst to carotene is used will be described. 100 cc. of cyclohexane, 50 cc. of glacial acetic acid, and 0.0828 gm. of platinum oxide catalyst were placed
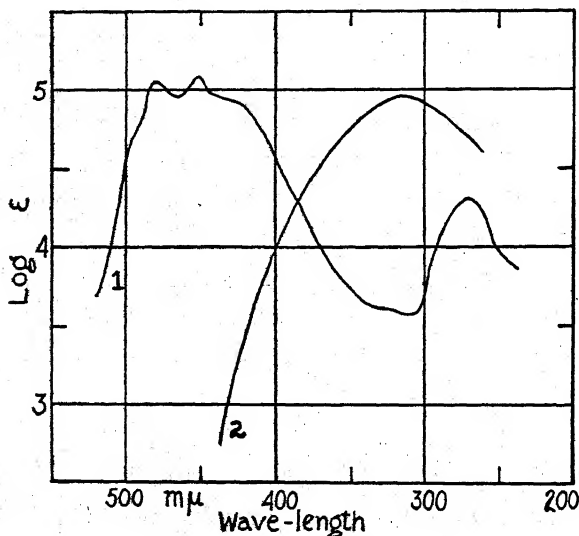
Fig. 1. Absorption spectra curves. Curve 1, carotene in cyclohexane solution; Curve 2, dihydrocarotene in ethanol solution.

in an Adams' catalytic apparatus[1] and the solvent saturated with hydrogen at 4 atmospheres pressure. 5.223 gm. of carotene were then added. No absorption of hydrogen occurred so 0.1264 gm. of fresh catalyst was added. Rapid absorption ensued, which ceased after 7.05 mols of hydrogen per mol of carotene had been absorbed. Addition of 0.0556 gm. of catalyst caused further absorption of hydrogen. At the end of 18 hours a total of 8.94 mols of hydrogen, corrected for the amount absorbed by the catalyst, had been added.

[1] Burgess Parr Company, East Moline, Illinois.

The reaction mixture was filtered from the platinum and washed free of acetic acid. The cyclohexane solution was dried, treated with charcoal, and filtered. The filtrate showed an optical rotation of $+0.78°$ in a 2 dm. tube.

By evaporating the cyclohexane, a viscous oily residue was obtained which distilled from a micro Claissen flask at 276° under 1 mm. pressure. The distillate was slightly yellow. It was decolorized by dissolving it in hot alcohol, cooling, and filtering off the colorless oil which separated.

The oil was dried at 60° in a vacuum oven, dissolved in carbon bisulfide, and tested for optical activity. The specific rotation was found to be

$$[\alpha]_D^{14} = \frac{+0.94 \times 50}{1.242 \times 5} = +7.56°$$

After removing the carbon bisulfide the product was again distilled. The analytical data on the distillate as well as the hydrogen absorption values indicate that the following reaction had occurred: $C_{40}H_{56} + 9H_2 = C_{40}H_{74}$.

$C_{40}H_{74}$. Calculated. C 86.55, H 13.45, mol. wt. 554.6
Observed. " 86.59, " 13.37, " " 589

Pregl's micro methods were used in making the elementary analyses. The molecular weight was determined by a micro Menzies-Wright method developed in this laboratory (to be published in Mikrochemie).

Density—$d\dfrac{29.2}{4} = 0.8828$, $d\dfrac{80.0}{4} = 0.8524$. These values were determined by means of a micro pipette, volume 34.09 c.mm.

Refractive Index—The index was determined by means of an Abbe refractometer. $n_D^{25} = 1.4869$, $n_D^{31} = 1.4848$, $n_D^{46} = 1.4792$. From these data an equation was formulated for temperature variation of refractive index.

$$n_D^t = 1.4962 - 0.000367t$$

The molecular refraction was found to be 180.3; that calculated from atomic refractions, 178.2, thus giving an exaltation of $+2.1$.

During the first distillation about 10 to 15 mg. of a crystalline solid collected in the receiver before the oil started to distil. This

material was found by analyses, neutralization equivalent, melting point, and molecular weight to be palmitic acid. The presence of this material at this place in the procedure shows how persistent an impurity may be, and indicates that in all work on the vitamin activity of carotene great precaution must be taken to guard against contamination.

High Ratio of Catalyst to Carotene. Eicosahydrocarotene

The next experiments were carried out with a large ratio of catalyst to carotene. The results showed that eicosahydrocarotene was formed according to the equation, $C_{40}H_{56} + 10H_2 = C_{40}H_{76}$. To use small quantities of material and yet to be able to distinguish between 9, 10, or 11 mols of added hydrogen a special apparatus had to be developed. The apparatus so designed gave results accurate to about 2 per cent, an accuracy sufficient for our purpose.

The apparatus was so designed and operated that the reaction vessel was brought to atmospheric pressure before hydrogen absorption began and kept at atmospheric pressure by "titrating" with hydrogen from a reservoir whose pressure could be accurately determined at all times. In this way no correction for vapor pressures had to be made. Compensating devices for temperature and pressure changes also eliminated uncertain corrections for these variables.

Into the reaction flask of the hydrogenation apparatus were put 1.5758 gm. of platinum oxide catalyst, 0.8518 gm. of carotene,[2] 150 cc. of cyclohexane, and 50 cc. of glacial acetic acid. 3 minutes after hydrogenation had begun the red color of the carotene had disappeared. When the absorption had seemingly stopped further additions of catalyst were made. Two such additions were made, 0.2384 and 0.2296 gm. respectively. 10 hours after the last addition, the reaction was stopped and the pressure readings observed. Calculation showed that 10.23 mols of hydrogen had been absorbed per mol of carotene.

The reaction mixture was filtered from the platinum, washed free of acetic acid with dilute sodium carbonate solution, and the

[2] M.p. 172°, twice recrystallized from chloroform and alcohol and dried at 40° for 4 hours and then at 18° for 12 hours at 0.001 mm. pressure.

cyclohexane removed by evaporation. A thick clear viscous oil was obtained which distilled at 206° at 10^{-4} mm. pressure.

The clear, colorless distillate had the following properties.

$C_{40}H_{76}$. Calculated. C 86.23, H 13.77
Observed. " 86.26, " 13.71

Optical Rotation—The rotations of the pure substance were observed in a micro polarimeter tube made of brass, with inside dimensions of 1.5 × 50.0 mm.

$$[\alpha]_D^{20} = \frac{+\ 0.148}{0.5 \times 0.8773} = +\ 0.337$$

Molecular Refraction—$n_D^{24.2} = 1.4821$, $d\frac{24.2}{4} = 0.8748$, $M_D = 181.42$. Calculated from atomic refractions $M_D = 180.32$. Exaltation observed $+\ 1.10$.

DISCUSSION

From the foregoing experimental results some possible deductions as to the structure of the carotene molecule can be made.

1. The specific rotations of carotene ($-63°$), dihydrocarotene ($+38.9$), octadecahydrocarotene ($+7.58$), eicosahydrocarotene ($+0.34$), and perhydrocarotene (approximately 0°) (3), imply that progressive hydrogenation tends to produce optical symmetry. To discuss all the potentialities inherent in these observations is outside the scope of this communication. Suffice it to say that, if perhydrocarotene is optically inactive, the two linkages hardest to saturate are in such positions as to produce asymmetry, which asymmetry may be removed by saturation. The most likely place for such unsaturation to exist is in an unsaturated or bicyclic ring.

2. Measurements of molecular refraction show exaltations for octadecahydrocarotene and eicosahydrocarotene of $+2.1$ and $+1.1$ respectively. Since these exaltations are much less than that corresponding to two and one double bonds respectively and of the same order of magnitude as that for cyclopropane rings the implication is that these hydrogenated derivatives contain such rings. The difficulty encountered in saturating these bonds and the change in optical rotation on hydrogenation tends to support the bicyclic ring hypothesis, but it is realized that certain types of

double bonds may show the same behavior. Work is in progress to distinguish between these two possibilities.

3. Since nine double bonds are easily saturated while the remaining ones are not, it appears probable that the nine are in a conjugated series.

4. The fact that carotene can be hydrogenated by means of aluminum amalgam favors the assumption that there are one or more rings in the carotene molecule attached to a chain containing a conjugated system of double bonds. This assumption is substantiated by Wislicenus' (11) statement that aluminum amalgam is not suitable for the hydrogenation of aromatic nuclei or double bonds in open carbon chains; and by Kuhn's (12) findings, that in the diphenyl polyenes only those unsaturated carbon atoms attached to rings are hydrogenated by this reagent.

5. From absorption spectra measurements on carotene and dihydrocarotene, it can be shown that the change in color produced by the addition of 1 mol of hydrogen is greater than that corresponding to the saturation of one double bond (*cf.* Kuhn (13)). Estimates indicate that three double bonds have been removed from the system of conjugated double bonds, one by saturation and two by separation. The color remaining in dihydrocarotene corresponds also to the presence of eight conjugated double bonds. From these observations then it appears that there may be a system of nine conjugated double bonds, conjugated further with two other unsaturated linkages, either double bonds or cyclopropane rings, and that this system is responsible for the color in the carotene molecule. In a recent paper Karrer (14) has also proposed such a conjugated system.

These conclusions support the structure of carotene previously proposed by the author (15).

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THE ACID-BASE EQUILIBRIUM OF THE BLOOD IN PATHOLOGICAL CONDITIONS

I. CHANGES OBSERVED IN THE TOXEMIAS OF PREGNANCY

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A number of observers (1–8) have demonstrated a lowering of the alkali reserve in normal pregnancy. As has been pointed out, this may be the result of one or the other of two factors acting separately or in combination; namely, an abnormal increase of acid in the blood or a withdrawal of base from the blood. That the pregnant organism may have an increased acid production was indicated by Hasselbalch and Gammeltoft (9). These workers found an increased ammonia nitrogen to total nitrogen ratio in the urine while the actual acidity of the urine decreased. Further, there was a lowering of the alveolar carbon dioxide tension. They considered that relative acidosis exists due to an increased acid production which is compensated by the decreased carbon dioxide tension.

The lowering of the alveolar carbon dioxide tension along with the demonstration by Marrack and Boone (4) and Gaebler and Rosene (6) of an increased pH of the blood would indicate a tendency to pulmonary hyperventilation. The slight lowering of the alkaline reserve may then, as suggested by Austin and Cullen (10), be considered a secondary effect of hyperventilation rather than as due to a true acidosis. Oard and Peters (7) object to this conclusion since the above pH determinations were made colorimetrically, a method, which as they point out has been subject to criticism, especially when applied to pathological bloods. By making a total acid-base equilibrium study of the serum of cases of normal pregnancy, Oard and Peters (7) found that there was no

change in the chloride or inorganic phosphorus. A reduction of the total base of about 8 mM occurred with an equal reduction of the anion content, consisting chiefly in a reduction of the serum bicarbonate and organic acid and to a lesser extent a decrease in serum protein. Oard and Peters concluded that no adequate explanation was available for the lowering of the alkaline reserve, although they observed that this was not the result of an actual accumulation of acid in the blood, but was associated with a reduction of the total base. This also seems to be the view taken by Stander, Eastman, Harrison, and Cadden (11).

During the last 2 years we have had the opportunity of making an acid-base balance study along the lines outlined by Peters, Bulger, Eisenman, and Lee (12) on cases termed "toxemias of pregnancy." These cases were all accompanied by hypertension (the systolic blood pressure varying from 150 to 223 mm. Hg) and proteinuria. It was thought that a study of abnormal cases might give some further information as to the cause of the lowering of the alkaline reserve.

Methods

The method of handling the blood has been described elsewhere (13). In all cases the blood was taken in the morning before breakfast. It was immediately separated into two fractions for whole blood and plasma analyses. The plasma was obtained without air contact and upon it the determinations of pH, CO_2 content, total protein, chloride, total base, and in some cases, phosphorus were made. The whole blood was employed for hemoglobin and non-protein nitrogen estimations.

The carbon dioxide content was determined by the method of Van Slyke and Neill (14). The Van Slyke procedure (15) for the estimation of chlorides was employed, although with the introduction of the modification by Wilson and Ball (16) the latter procedure was used. The pH was determined colorimetrically and in a few cases electrometrically as described by Myers and Muntwyler (17). For the total base estimation the method of Stadie and Ross (18) was employed. The phosphate was not determined in all cases. For this reason the total base figures were not corrected for the loss by combination with phosphate. Since the phosphate is not altered in normal pregnancy, and further

since in the few determinations made here a normal phosphate concentration was encountered, only a small error in base could be introduced by this omission. The oxygen capacity was determined by the method described by Van Slyke and Neill (14). For the determination of total protein, 0.5 cc. of plasma was subjected to a Kjeldahl procedure. From the total nitrogen 30 mg. per 100 cc. was subtracted. This is to correct for the non-protein nitrogen since in some cases the non-protein nitrogen was not determined on the same sample of blood employed for the acid-base determinations.

Calculations

The value for per cent of total protein was converted into mM of base combined with protein by the formula of Peters *et al.* (19).

$$Bp = 1.072 \ P(pH - 5.04)$$

in which an albumin : globulin ratio of 1.8 was assumed. Bp and P represent respectively the mM of base combined with the protein and the per cent of protein.

The mM of base combined as $BHCO_3$ was obtained from the pH and total CO_2 by the following equation.

$$mM \ BHCO_3 = CO_2 - \frac{CO_2}{(10^{pH-6.10} + 1)}$$

in which CO_2 is the mM concentration of total CO_2.

The total acid in this report is represented as: T.A. $= BHCO_3$ $+ BCl + BP$ where $BHCO_3$, BCl, and BP represent mM of base combined with HCO_3, Cl, and P respectively. The difference between the total base determined and the total acid is termed the undetermined acid and represents the amount of base present as phosphate, sulfates, and salts of organic acids.

Observations

The results of the analyses are collected in Tables I and II. Table I presents a comparison of the electrometric pH observed at 38° with the colorimetric pH observed at room temperature (about 25°) and corrected to 20° on plasma obtained from six cases of toxemia of pregnancy. The samples were obtained after delivery in Cases 1 and 6. With the other cases a blood sample was

obtained both before and after delivery.   An average C correction of 0.223 pH was obtained on the ten comparisons.   The pH values recorded in Table II were obtained colorimetrically with 0.22 pH as the C correction.   A C correction of 0.22 was employed since, in a previous comparison of the electrometric and colorimetric methods on blood plasma having in the majority of cases about the same pH range as those observed here, an average C correction of 0.224 was obtained.   Further, with subsequent work which is to be published in the near future, it was found that the C correction

TABLE I

Comparison of Colorimetric and Electrometric pH of Plasma in Cases of Toxemias of Pregnancy

Case	Date	pHe 38°	(pH)20°*	C correction	CO₂ content	Plasma Cl as NaCl	Inorganic P	Urea N	Non-protein N	Date of delivery
	1928				*vol. per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	
1. A. Z.	Feb. 20	7.50	7.73	0.23	57.9		3.0	5.8		Feb. 16
	" 27	7.49	7.69	0.20	65.5	612	3.6	9.3	33.4	
2. R. H.	" 20	7.52	7.76	0.24	56.0		2.9	8.5		Feb. 17
	" 27	7.48	7.68	0.20	54.1	615	3.3	7.2	31.3	
3. C. S.	" 27	7.45	7.67	0.22	50.4	638	4.6	7.5	31.4	Feb. 28
	Mar. 1	7.44	7.67	0.23	53.2	625	3.5	16.2	33.6	
4. D. G.	" 5	7.48	7.69	0.21	54.8	612		9.8	20.2	Mar. 18
	" 29	7.46	7.68	0.22	64.5	600	3.8	10.3	33.3	
5. K. H.	" 22	7.40	7.64	0.24	43.1	638	2.6	10.6	32.3	Mar. 20
6. M. P.	" 29	7.42	7.66	0.24	50.4	625	3.4			" 30

* Corrected to 20° by adding 0.01 pH for every degree the solution temperature was above 20°.

of the sample with a given protein concentration varies with the pH range.   The higher the pH of a given sample the greater the C correction.   Of the twenty-six cases reported only one case, Case 7, showed a pH below 7.40.   With bloods obtained ante partum, thirteen cases had a pH of 7.48 or above.   Nine cases showed a higher pH post partum than ante partum.   The highest pH value obtained was 7.54 observed with Cases 20, 24, and 25, the three samples being obtained post partum (4 to 11 days).   The average antepartum pH value found was 7.47.

The plasma bicarbonate concentration was always found lowered with bloods obtained ante partum. Considering the twenty cases presented in Table II, where in most instances blood was obtained before and after delivery, the highest plasma bicarbonate concentration observed ante partum was 24.2 mM, the lowest 16.5 mM, and the average 20.9 mM. After delivery the bicarbonate always increased with a maximum observed increase in Case 17 of 8.5 mM in 10 days post partum. The average postpartum bicarbonate concentration was 25.3 mM.

The chloride concentration of the plasma, with the exception of Cases 8, 10, and 11 (Table II), was 100 mM or above in the antepartum samples. With the exception of Cases 10 and 16, where observations were made before and after delivery, the chloride concentration was decreased post partum. In some of these cases there was an almost equivalent increase in the bicarbonate associated with the decrease in chloride. The highest plasma chloride encountered ante partum was 112.1 mM, the lowest 92.5 mM, and the average 102.8 mM. The average postpartum chloride was 100.8 mM.

The total acid concentration (total acid $= BHCO_3 + BCl + BP$) varied from 145.3 mM to 136.1 mM ante partum with a tendency to increase in more than half of the cases observed post partum to give a maximum observed total acid value of 147.5. The average antepartum total acid finding was 141.4 mM while the average postpartum finding was 144.0. The values of the total base obtained ante partum varied between 139.2 and 156.6 mM, with an average of 144.7 mM. Of the thirteen total base determinations made ante partum, eleven varied between 140.1 and 148.1 mM. The postpartum total base values averaged somewhat higher than the antepartum, namely 148.7 mM.

DISCUSSION

As can be seen from Table II there is no elevation of urea nitrogen and hence the acid-base findings are not complicated by changes dependent upon nitrogen retention. At the bottom of Table II are given the maximum, minimum, and average values for the electrolyte changes observed ante partum along with the average postpartum findings. As mentioned above, it has been recognized for some time that normal pregnancy is accompanied by a lowering

TABLE II

Acid-Base Balance in Toxemias of Pregnancy

Case	Date	pH	CO₂	Plasma HCO₃	Cl	Protein	Total acid	Total base	Total base – total acid	O₂ capacity	Urea N	Date of delivery
			mм	mм	mм	mм	mм	mм	mм	vol. per cent	mg. per 100 cc.	
	1938											*1938*
7. O. M.	Sept. 15	7.39	22.43	21.3	101.9	18.1	141.3	156.6	15.3	16.9	9.6	Oct. 10
	Oct. 15	7.46	29.13	27.9	96.4	18.5	142.8	152.0	9.2	16.8		
8. O. W.	" 14	7.46	18.65	17.9	94.5					21.6	14.4	Nov. 3
9. M. R.	" 15	7.40	18.85	17.9	100.5	18.5	136.9	147.6	10.7	16.5	10.9	" 4
10. L. S.	" 22	7.45	25.18	24.1	92.5			139.2		21.4	15.0	Oct. 23
	" 29	7.51	26.17	25.2	99.1					21.6		
11. M. H.	" 22	7.41	23.56	22.4	98.6			148.1		15.3	7.8	Oct. 23
	" 29	7.53	28.14	27.1	93.5			148.0		18.4		
	1939											*1939*
12. L. K.	Feb. 10	7.49	25.24	24.2	100.9	14.6	139.7	141.5	1.8	17.4	6.0	Feb. 13 Cesarean
13. A. B.	" 25	7.44	24.66	23.6	104.0	17.2	144.8	144.4	0.6	15.1	5.0	Mar. 12
	Mar. 20	7.45	26.91	25.7	102.0	16.1	143.8			11.4		
14. J. S.	Feb. 26	7.52	22.97	22.1	102.6	20.6	145.3			13.5	6.7	Mar. 28
15. V. F.	" 27	7.48	23.05	22.1	102.6	15.5	140.2			12.3	10.7	" 9
	Mar. 15	7.46	24.93	23.9	101.4	15.4	140.7					
16. I. J.	Apr. 3	7.46	20.65	19.8	101.8	18.6	140.1	140.3	0.2	14.6	9.6	Apr. 4
	" 17	7.49	23.36	22.5	106.9	18.1	147.5			13.7		
17. R. C.	" 15	7.43	17.24	16.5	112.1	15.2	143.8	142.0		15.0	7.1	May 1
	May 11	7.47	26.06	25.0	103.8	16.3	145.0	155.4	10.4	19.0		
18. N. S.	Apr. 16	7.48	21.51	20.6	109.5	14.8	144.9	143.8		15.6		Apr. 16
	" 30	7.44	25.33	24.2	106.1	17.2	147.5	147.6	0.1	12.0		

Case	Date											Delivered
19. P. S.	Apr. 22	7.46	21.33	20.4	109.9	14.8	145.7	150.9	5.2	10.3	7.8	May 4
	May 10	7.44	29.83	28.5	102.4	18.0	142.1	142.6		5.6	5.2	
20. C. H.	Feb. 3	7.49	22.48	21.6	102.5	18.1	146.3	140.1	0.5	16.1	14.9	Feb. 2
	" 8	7.54	22.52	23.0	104.6	15.8	140.1	147.2		11.8	9.2	
21. V. B.	" 15	7.48	22.21	21.3	103.0	19.3	145.9	146.2	1.3	12.0	11.6	Feb. 18
	Mar. 1	7.51	25.75	24.8	101.8	14.3	139.5	145.8		10.3	10.3	
22. G. B.	" 1	7.51	21.20	20.4	104.8	16.2	145.3	148.2	6.7	15.7	10.0	Mar. 13
23. M. D.	May 1	7.52	21.82	21.0	108.1	18.4	142.5	142.7	0.5	18.6	14.8	May 8 Cesarean
	" 24	7.52	27.00	26.0	98.1	16.7	142.9	140.8		14.7	14.7	
24. M. G.	Apr. 24	7.53	22.74	21.9	104.3	18.0	141.3	152.8	5.7	17.8	8.8	June 24
	July 5	7.54	28.00	27.0	96.3	18.4	140.8	146.8		18.5	11.6	
25. E. W.	Apr. 24	7.49	21.67	20.8	101.6	15.9	139.0	13.8		11.6		May 20
	May 24	7.54	25.44	24.3	98.8	13.3	136.1			21.4		
26. M. M.	" 27	7.52	22.40	21.4	101.4	13.3			10.7		11.6	Not delivered to date
Summary of antepartum findings												
Maximum		7.53	25.24	24.2	112.1	18.6	145.3	156.6				
Minimum		7.39	17.24	16.5	92.5	13.3	138.1	139.2				
Average		7.47	22.68	20.9	102.8	16.5	141.4	144.7				
Average postpartum findings		7.48		25.3	100.8	17.2	144.0	148.7				

of the blood bicarbonate. The results recorded in Table II show that this is also true with the cases of toxemia of pregnancy studied. In fact the average antepartum findings in these cases agree well with the values recorded in Table III presented by Oard and Peters (7). The average plasma bicarbonate concentration observed in these cases is 20.9 mM as against 22.1 mM reported by Oard and Peters. In the series of cases observed by the latter authors the total acid and total base concentrations are slightly higher than those reported here. The discrepancy is practically made up for when the average mM of base combined as phosphate is added to the total acid concentration given in Table II and the total base values corrected for base lost in combination with phosphorus.

The average postpartum findings are of interest. The bicarbonate and protein showed an average increase of 4.5 mM and 0.7 mM, respectively, while the chloride was decreased on the average by 2.0 mM. The total acid concentration showed an average increase of 2.6 mM while the total base increased 4.0 mM.

If one considers the value of 153.8 mM of total base, given by Oard and Peters, as the average normal serum concentration, it is at once apparent that there was a loss of base in these cases. With this decrease in base there was also a tendency to a definite increase in pH. The maximum antepartum pH finding was 7.53 with an average of 7.47. This would indicate that hyperventilation was a factor in the bicarbonate decrease observed. This supposition is further supported by the decrease in chloride post partum associated with the increase in bicarbonate. As was mentioned previously, the assumption of hyperventilation as the main cause of the bicarbonate decrease is in agreement with the views of Austin and Cullen (10) who based their opinion on the increased pH observed by Marrack and Boone (4), and with the views of Gaebler and Rosene (6), who found an increased plasma pH ante partum which returned to normal with the bicarbonate post partum.

Oard and Peters (7) object to the view that hyperventilation is the causative factor in the lowering of the alkali reserve, in the first place because the pH determinations of Marrack and Boone (4) were done colorimetrically, a method which is open to criticism and further since neither Hasselbalch and Gammeltoft (9), nor

Menten (20), or Williamson (5) observed an increased pH in pregnancy. It should perhaps be noted that the methods employed by Menten and by Williamson were also not sufficiently delicate to have given an answer to this question. Oard and Peters further point out that Peters, Bulger, Eisenman, and Lee (21) found that in a case of chronic hyperventilation, the total base always remained within the normal limits of 152 to 160 mM, a situation not encountered in pregnancy.

It is true that the colorimetric method is open to some criticism especially when the estimation is performed at 20° and corrected to 38°. As was pointed out by Austin, Stadie, and Robinson (22) and as has been verified in this laboratory (observations to be published in the near future), the Cullen correction for plasma with a given bicarbonate concentration varies with the pH range and the protein concentration. Consequently the higher the pH with a given protein and bicarbonate concentration, the greater the C correction; and, the lower the protein concentration with a given pH and bicarbonate concentration, the lower the C correction. Since in pregnancy there is a lowering of the protein concentration and also an increase of pH these two factors tend to oppose each other in affecting the C correction so that a relatively constant C correction may be expected. This is just what was found in the series of ten samples of plasma obtained from cases under observation recorded in Table I. The C correction averaged 0.223. It would seem that this correction should give pH values quite close to the true pH when applied to the cases reported in Table II, since the pH range and variations in protein concentration were within quite narrow limits. In agreement then with Marrack and Boone (4) and Gaebler and Rosene (6) the pH was generally found to be above the normal in pregnancy. Contrary to Gaebler and Rosene the pH value had in the majority of cases increased slightly when the postpartum observations were made. The reason for this is not clear, although it should be noted that the second blood specimen was taken quite soon after delivery. It is also possible that in cases of toxemia a longer period is required for the acid-base balance to return to normal than in cases of normal pregnancy. At any rate it is apparent from the observation reported, that some little time must elapse post partum before the normal acid-base balance is restored. Attention should be called to the results of Stander,

Eastman, Harrison, and Cadden (11) who concluded from electrometric determinations that the pH in pregnancy is not changed from the non-pregnant value. This conclusion is based on three samples from non-pregnant women and three samples from cases of normal pregnancy. It should be noted that the average pH of the samples from the cases of pregnancy is 7.37 as opposed to 7.34 for the normal.

In a case of chronic hyperventilation Peters, Bulger, Eisenman, and Lee (21) found that the total base remained within normal limits. If these observations could be applied to all cases of chronic hyperventilation, a lowered plasma total base could not be explained on this basis. However, this was but a single case and it would seem that further observations are required before it can be concluded that hyperventilation does not disturb the total base of the plasma, since it has been shown by Collip and Backus (23) and Grant and Goldman (24) that in hyperpnea the fall in plasma CO_2 is associated with a marked decrease in urinary acidity. Shohl (25) has also made the interesting suggestion that the decrease in total base may be associated with the increase in the fetal requirements. Although it is improbable that this is the chief factor concerned, it should not be altogether ignored.

In agreement with Oard and Peters, however, it has been found that the decrease in bicarbonate is not the result of an actual collection of abnormal acids in the blood, but is associated with a lowered base.

SUMMARY

1. An acid-base equilibrium study of the plasma of twenty-six cases of toxemia of pregnancy is reported.

2. A lowering of the alkaline reserve (to an average of 20.9 mM of plasma bicarbonate concentration) was found, as has been observed in normal pregnancy.

3. The pH was elevated to 7.48 or above in thirteen of the twenty-three cases studied ante partum, with an average pH of 7.47.

4. The total base concentration was decreased from the average normal of 153.8 mM, as given by Oard and Peters, to 144.7 mM (uncorrected for loss due to phosphorus).

5. Post partum the findings showed on the average a considerable increase in the bicarbonate and total base, with a slightly smaller increase in total acid and a slight increase in protein and pH. There was a small decrease in chloride.

6. The lowered plasma bicarbonate (so called acidosis of pregnancy) was not due to an accumulation of abnormal acids.

7. From the data at hand it would seem most logical to conclude that the fall in the plasma bicarbonate noted in the present series of cases of toxemia of pregnancy (and in normal pregnancy) is due to hyperventilation, and that the fall in total base is a further compensatory phenomenon.

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A CRITIQUE OF THE LINE TEST FOR VITAMIN D

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In spite of the extensive use of the line test for vitamin D, there
has been no critical study of its suitability for precise estimations.
It is generally assumed that biological assays are subject to such
errors that at best they are a poor, though unavoidable, substitute
for other methods. In our studies on the production of vitamin D
by irradiation it was early apparent that physicochemical methods
of estimation could not replace the biological, yet that such prob-
lems as the identification of the pure vitamin required improved
assay technique.

The original description of the line test by McCollum, Sim-
monds, Shipley, and Park (1) is the basis of its many modifications.
Our application of it is a refinement in detail, developed from the
data obtained in a very large number of assays. A year ago, when
10,000 such records had been collected, we determined by graphic
analysis certain numerical relations between the degree of healing
produced and the dosage which produced it. During the past
year we have checked the reliability of the graphic interpretations
with 5000 additional assays. We are confident now that the line
test may be applied as an analytical procedure with a degree of
accuracy hitherto considered impossible.

Breeding and Care of Rats

Our rats are the inbred Wisconsin laboratory strain used by
McCollum and by Steenbock. They are kept in metal cages,
bedded with sterilized shavings. The temperature in the ratteries
is held at 20–25° throughout the year. Breeders are selected by
weight from the young weaned each week, so that the age average
of the parents is constant. Under these conditions there is no

discernible seasonal variation in fertility. Small litters (up to eight) are not reduced, but litters of more than eight are reduced to six. This lightens the burden of nursing on the mothers subjected to heaviest reproduction. It is often feasible to suckle some of the young from the large litters by foster-mothers having small litters of their own.

The parent rats are kept on the stock diet of Steenbock, slightly modified as follows:

Yellow maize	57.0
Whole milk powder	25.0
Linseed oil meal	12.0
Crude casein	3.7
Alfalfa leaf meal	1.5
Iodized table salt	0.4
Calcium carbonate	0.4

With tap water, *ad libitum*

Production of Rickets

At the age of 24 days the young are weaned. They weigh on the average 50 gm., but their exact weight is unimportant. They are removed to a room from which all daylight, *even that filtered through glass*, is excluded, and kept for 18 to 24 days at 20–25° on McCollum's Diet 3143, compounded as follows:

Whole wheat flour (Pillsbury's "100 per cent")	33.0
Yellow maize (select quality, finely ground)	33.0
Wheat gluten (Pure Gluten Food Company)	15.0
Gelatin powder (Coignet Super-X)	15.0
Calcium carbonate (c.p.)	3.0
Sodium chloride (c.p., powder)	1.0

With tap water, *ad libitum*

Although most of our work has been done with Diet 3143, we have investigated several other promising rations. None gave better results than Diet 3143. A palpable enlargement of the wrists and a slightly waddling gait usually indicate that rickets has developed to the proper degree. The rats should not be kept on the rickets ration until they are definitely weak. Such animals may exhibit anorexia, or they may fail to neaten themselves as evidenced by an ophthalmia due to the eyes being stuck with debris.

The rickety rats are put on test in individual cages.   It is well to so arrange the animals that those used in a given assay do not all come from the same litter.   The fewer the litter mates, the less will be the error from litter variation.   One control rat is taken from each litter represented.

In our standard technique the substance to be tested is incorporated in Diet 3143 by trituration.   The test preparation is administered for exactly 5 days, an individual daily food consumption record being kept.   Each rat should eat not less than 2 gm. in any one day and not less than 4 gm. per day for the period, and should not lose weight.   We have observed that animals being cured of rickets may for a few days gain less than either normal or rickety animals, and that occasionally healing may be followed by a temporary loss of weight.   But on the other hand, a loss of weight, with presumably the liberation of tissue phosphate, may itself sometimes occasion calcification.   Inasmuch as there is no means available for distinguishing between these causes and effects, it is imperative to discard all rats which lose even as little as 1 gm.

The autopsy is performed in accordance with Shipley's procedure, which consists in exposing longitudinally sectioned halves of the proximal end of the tibia in 2 per cent $AgNO_3$ to intense direct illumination under a low power binocular microscope. Upon the calcified areas silver phosphate is formed and reduced to black silver.[1]   The principal criteria of healing are the development of the line at the zone of provisional calcification and the reappearance of bony trabeculæ in the metaphyseal osteoid.   Either leg may be sectioned, as the rachitic lesions exhibit bilateral symmetry to a remarkable extent.

Four Degrees of Healing

At McCollum's laboratory in 1923 one of us (C. E. B.) devised a scale of healing for the line test which graded the observed silver deposits into four groups: $+$, $++$, $+++$, and $++++$. Originally intended as a convenience in recording data, these arbitrary grades bore no predetermined relation to each other.

[1] The recent study by Cameron indicates that the silver stain is not specific for calcium or phosphorus.   The reader should refer to this important work of Cameron, G. R., *J. Path. and Bact.*, **33**, 929 (1930).
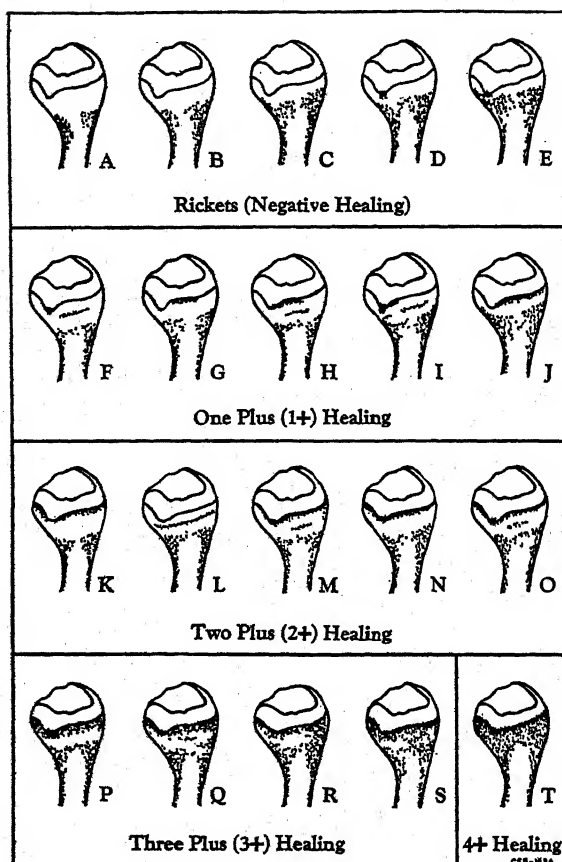
FIG. 1. *A*, *B*, *C*, *D*, and *E* are examples of rickets. *A*, extremely severe rickets, as seen in animals which have received Diet 3143 for a prolonged period; *B*, severe rickets; *C*, moderately severe rickets; *D*, moderately severe rickets with a spot of old calcium evident in the principal tongue of cartilage; *E*, the poorest type of rickets that can be accepted in controls. *F*, *G*, *H*, *I*, and *J* are examples of 1+ healing. *F*, very slight healing evidenced by a metaphyseal line; *G*, very slight healing evidenced by a trace of calcium at the distal edge of the cartilage; *H* and *I*, slight healing; *J*, strongest 1+ healing. *K*, *L*, *M*, *N*, and *O* are examples of 2+ healing. *K*, moderate epiphyseal healing; *L*, moderate metaphyseal healing; *M* and *N*, stronger healing; *O*, strongest 2+ healing. *P*, *Q*, *R*, and *S* are examples of 3+ healing, in order of increasing calcification. *T* shows 4+, or complete healing.

Obviously, 4+ healing indicated greater antiricketic action than 1+, but the relation was certainly not 4:1. Several years ago we published (2) colored illustrations of the different grades, done by the medical artist, Didusch. These standards served as the basis of the present investigation.

It is unfortunately true that not all rats give line tests which look exactly like one or another of the standards. In fact, one finds innumerable gradations. To recognize more than four degrees would seem to be splitting hairs, so to obtain the desired finesse in the recording of observations we have adhered to the four standards, but have prepared a series of supplementary sketches to illustrate the limits of each (Fig. 1).

The sketches in Fig. 1 show not only different degrees, but also different types of healing. The epiphyseal type, wherein the new calcium is laid down in a more or less linear arrangement at the distal edge of the cartilage, is the most frequently observed. However, for reasons that are not understood, the deposition may occur first in the metaphysis (metaphyseal healing), and this type may quite properly be considered in the line test. It is probable, also, that in some animals the first recalcification is diaphyseal, and contiguous with the trabeculæ that remain after rickets develops. No sketches were made to illustrate this type, which is, of course, useless for the line test.

Quantitative Relations between Healing Grades

In reading many line tests we sensed the existence of a relationship between the grades of healing and the dose of antiricketic material administered. Clearly, it would be of the utmost usefulness to place this relationship upon a numerical basis. This was done in the following manner.

We examined the protocols of 10,000 rats upon which the line test had been performed. The antiricketic agent in about half of these assays was cod liver oil; in the remainder, irradiated sterols, various fish oils, and miscellaneous materials. For one reason or another many of the records had no statistical value, and they were therefore rejected. We succeeded in selecting about 4000 protocols in which the healing agent was administered at two effective levels, the one being double the other. For example, several hundred samples of cod liver oil were administered at $\frac{1}{3}$ per

cent and $\frac{1}{4}$ per cent, or at $\frac{1}{4}$ per cent and $\frac{1}{2}$ per cent in Diet 3143, as a part of our routine procedure for grading medicinal oil. The number of animals involved in these pairs of assays ranged from two to more than twenty. Where only two were used; *i.e.*, one rat for each of the two levels, the degree of healing recorded was $1+, 2+, 3+,$ or $4+,$ as the case might be. But where more than two rats were used the total score in plusses was divided by the number of animals, giving in most instances fractional averages. Thus $\frac{1}{4}$ per cent of a particular sample of cod liver oil might be found to induce $+, -, +++, ++,$ and $+$ healing when tested with five rats. The total score is $7+,$ which divided by 5 equals $1.4+$ for the series. In like manner it might be found that double this dosage, *i.e.* $\frac{1}{2}$ per cent, tested with four rats, induced $++,$ $+++, +++,$ and $++$ healing or a total of $10+.$ The average is $10+ \div 4 = 2.5+.$

And so on, with the 4000 protocols, we tabulated two sets of figures, to the first decimal place, the one showing the degree of healing induced by the various agents at a given level, and the other showing opposite it the healing induced by doubling the dosage. Each pair of figures was weighted according to the number of animals represented. Having completed this tabulation, we gathered together all the pairs in which the lower level of dosage induced $0.1+, 0.2+, 0.3+, \ldots 3.9+,$ and opposite each of these sets of subtotals we recorded and averaged the degree of healing induced by the double dosage.

We then plotted on coordinate paper the weighted pairs of values, so as to obtain a curve (Fig. 2, Curve A) which indicates at a glance the degree of healing obtained by doubling the dose of antiricketic agent giving observed degrees of healing at the lower level. In Fig. 2, Curve A, the observed healing, in plusses, is indicated on the vertical axis; the effect of doubling the dose is read, in plusses, on the horizontal axis. It should be noted in passing that the points which described the curve did so with remarkable exactness—very few points fell more than a mm. from the curved line, so that the curve required little estimation or interpolation on the part of the draftsman.

Fig. 2, Curve A, has little practical usefulness by itself, but it is the basis of further graphic derivations which are of the greatest importance in interpreting the line test.

Cod Liver Oil as Potency Standard

Various methods have been proposed from time to time for the standardization of vitamin D preparations. Essentially they all consist in measuring the response of rats to the test substance in comparison with the response to an actual or hypothetical standard. Since no system of expressing potency in units per gm. is entirely satisfactory, we have adhered to the simple practice of comparing unknowns to average cod liver oil, and expressing the potency as a cod liver oil coefficient. This may be objected to on the ground that cod liver oils vary in potency, but the objection is
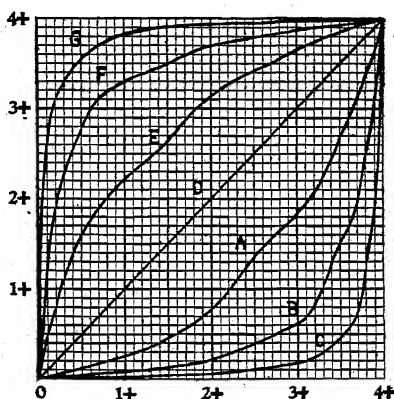
Fig. 2. Effect on healing of increasing dosage by different multiples: Curve A, 2; Curve B, 4; Curve C, 8; Curve D, 1; Curve E, ½; Curve F, ¼; Curve G, ⅛.

unimportant in view of the fact that the potency of *average* cod liver oil has been accurately determined.

It is true, as we have shown in collaboration with Hess (3), that the vitamin D potency of the liver oil of *individual* codfish may vary as much as 1000 times. However, the cod liver oil of commerce represents, even in small batches, the average of vast numbers of individual fish. Several years ago we pooled together 225 samples of Newfoundland oil and forty-four samples of Norwegian oil, each of known history, and subjected the mixture to assay, using forty rats. Happily, the mixture gave exactly 2.0+ healing when administered at ¼ per cent in Diet 3143. The same value was obtained with the Newfoundland and Norwegian

mixtures separately. *Average cod liver oil was therefore defined as that which induces 2+ healing when administered to rickety rats at $\frac{1}{4}$ per cent in Diet 3143 for 5 days.* The variation in vitamin D potency of commercial cod liver oil is less than is commonly supposed. One seldom finds a pure specimen which is more than twice, or less than half, as potent as the average, and by mixing several samples, a standard closely approximating the average in potency can readily be prepared. However, no standard solution is necessary in practicing the line test. It is merely necessary to determine the dilution of the unknown which will give exactly 2.0+ healing in 5 days.

Graphic Determination of the 2+ Dose

It is evident, from Fig. 2, Curve A, that the line test is most critical between the 1+ and 2+ healings. This follows from the fact that the curve is least tangential in this region. Nevertheless, good sensitivity is evident between 0.3+ and 3.0+. Since we have taken as the basis of all comparisons the healing produced by average cod liver oil—by definition, 2.0+, it is now highly desirable to devise means of translating any observed degree of healing into the 2+ basis. For example, suppose a solution of activated ergosterol gave 2.9+ healing. How much would this solution have to be diluted in order to give exactly 2.0+? Or, stated differently, how much less of this solution, without further dilution, would have to be administered in order to give exactly 2.0+? Until now, such information could only be guessed at, or at best determined by the extremely clumsy method of trial and error, requiring a great number of animals. We have developed graphs for this purpose, as follows:

It occurred to us that Fig. 2, Curve A, could be manipulated so as to yield information which at first glance one would not think it possessed. As explained above, this curve indicates the degrees of healing produced by doubling the doses which gave observed degrees of healing. Now, by simply redoubling the healing degrees on the vertical axis, using this curve, we obtained a second curve, Fig. 2, Curve B, which shows the effect of quadrupling the original dose. For example, if a given dose produced 0.7+ healing, Curve A tells us that doubling this would produce 1.9+. If this in turn is doubled, Curve A indicates that 3.16+ would result, and thus we

have a point for Curve B. All the other points were obtained by
the same device. Similarly, by twice redoubling, we obtained
Curve C, which shows the effect of increasing the original dose
eight times.

Now by drawing the diagonal, D, we obtained a curve which
indicates the effect of multiplying the original dose by 1, or in
other words, not changing it at all. Next, by drawing reciprocal
curves of Curves A, B, and C, we obtained Curves E, F, and G,
which indicate the effect of multiplying the original dose by $\frac{1}{2}$, $\frac{1}{4}$,
and $\frac{1}{8}$, respectively.
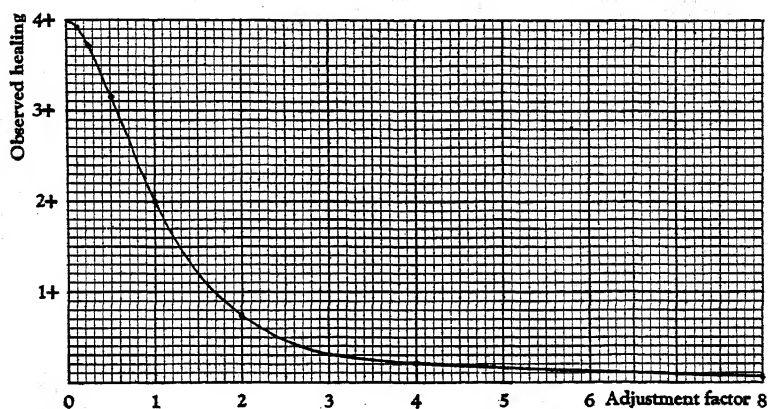
FIG. 3. Adjustment of potency required to give 2+ healing

In these seven curves, showing the effect on healing of increasing
the original dose by seven different multiples, we have data from
which a new curve can be constructed so as to show the effect of
increasing by multiples of any magnitude (Fig. 3). Fig. 3 was
obtained by plotting the points of intersection of the vertical 2+
line of Fig. 2 with Curves A, B, C, D, E, F, and G, these points
being indicated by dots on the new graph.

Fig. 3 shows (on the horizontal axis) the dilution or fortification
which is required by any preparation giving an observed degree of
healing (on the vertical axis). For example, a certain solution of
activated ergosterol gave 2.9+ healing (vertical). The curve
shows that by diluting this solution to six-tenths (horizontal) of
its former strength, the resulting product would give exactly 2.0+

healing. Or again, a 1.2+ solution would need to be made 1.5 times as strong, in order to give 2.0+ healing. Obviously this curve, like the curves from which it was derived, is most accurate where it is least tangential.

Since Fig. 3 is used for adjusting to a desired potency, it finds its principal application in the preparation of commercial activated ergosterol solutions. In research work, on the other hand, one most frequently desires to know the exact potency of a particular solution. To this end, we have replotted the data of Fig. 3 in the form of Fig. 4. In Fig. 4 one locates the observed healing in plusses on the vertical axis, and reads on the horizontal axis the exact potency of the substance in multiples (or fractions) of
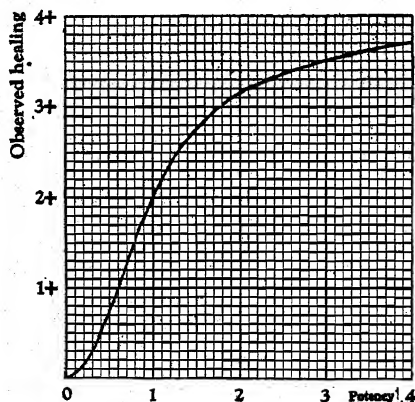
FIG. 4. Potency equivalent of observed healing

standard, 2.0+, potency. For example, the cod liver oil coefficient of the solution which gave 2.9+ healing was 1.64, and that of the solution which gave 1.2+ was 0.67.

Probable Error of Assay

So far as we are aware, no attempt has been made to estimate the error to which assays for vitamin D are subject. The smoothness of Fig. 2, Curve A, suggests that when many rats are used, the error is trivial. It is more important, however, to estimate the error experienced with fewer rats, and to gain some idea as to how many rats must be used to attain a desired exactness.

A solution of irradiated ergosterol in oil was made up so as to have a cod liver oil coefficient of roughly 100. It was then diluted 1:100 and tested at $\frac{1}{4}$ per cent on 200 rats—100 male and 100 female. The animals were typical of our stock, and were taken during 3 months in the spring of 1929. Those which failed to meet the requirements of growth or food consumption during the 5 day test period were, of course, replaced by others. Individual records were kept of the weight at the beginning and end of the test, the daily food consumption, and the observed healing.

At the outset of the statistical study, it was seen that a deviation from the classical method for computing probable error was necessitated by the fact that the healing degrees in plusses bore no direct numerical relation to each other. On account of the occurrence of the negative and 4+ extremes of healing, which are not translatable by the graphs into terms of potency, the best we could do was to average the plusses, and then deal with the averages. We did this, keeping in mind the concept of probable error as the "limits within which it is as likely as not that the truth will fall."

The average degree of healing for the 200 rats was 2.30+. Fig. 4 translates this to indicate that the potency of the ergosterol solution was actually 116 ×. The potency as determined with 100 male rats was 119 × (Δ + 2.6 per cent), and with 100 female rats, 113 × (Δ − 2.6 per cent). The potency as determined with forty albino rats, twenty of each sex, was 114 × (Δ − 1.7 per cent), and with 160 colored rats, eighty of each sex, 117 × (Δ + 0.9 per cent). In all these sets, the deviation from 116 × was less than the probable error (see below) for the number of animals used. Hence we may state that *neither the sex nor the color of the rats is an important factor in the line test.*

In briefest outline, the data from the 200 line tests were worked up as follows: By throwing dice, and by other purely arbitrary methods, the 200 protocols were rearranged into eight groups of 100 rats each, 8 groups of 50 each, 10 groups of 40 each, 20 groups of 20 each, 20 groups of 10 each, 50 groups of 4 each, and 100 groups of 2 each.

Each group was totalled and averaged as to healing degrees in plusses, and each average was translated by Fig. 4 into potency. The deviation from 116 × (the grand mean), was computed in per cent for each group in each set of groups. The deviations were

added, and the sums were divided by the number of groups in each set. Thus we obtained mean deviations from the grand mean, corresponding to the number of rats used.

Naturally, the individual group deviations were most variable where the number of rats in each group was smallest. The smaller groups therefore were made more numerous, in order to minimize error in the mean deviations. That the grouping of the tests was judiciously enough performed, to warrant the assumption that the mean deviations represented the probable errors, is indicated by the fact that the mean deviations were never exceeded in more than 50 per cent of the groups in any set of groups. In one set the
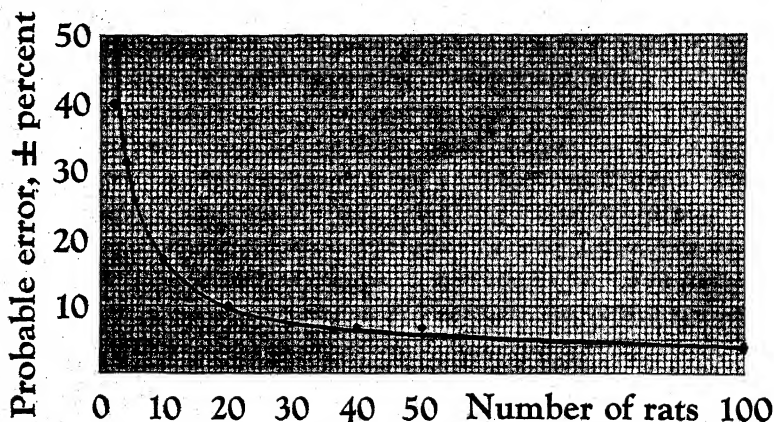
Fig. 5. Relation of error to number of rats

mean deviation was exceeded by only 25 per cent of the groups, and in the average of all sets, by 35 per cent.

These deviations, which we consider the probable errors, are graphically illustrated in Fig. 5. It is interesting to note that the curve is asymptotic—that by the use of 100 rats for an assay the probable error is reduced to 4 per cent, and that a much greater number of rats would not reduce it much more. With twenty rats the error is 10 per cent, with ten rats, 17 per cent, and with four rats, 31 per cent. With fewer than four rats the error rapidly becomes great.

One is tempted to apply the classical method of determining probable error to the deviations from 116 × as computed for each group in each set of groups. The values so determined are much

smaller than those shown in Fig. 5. For instance,' the probable error for a twenty rat assay would appear to be only 2 per cent, as against the 10 per cent which we give. This is undoubtedly fallacious, although we have not been able to ascertain the origin of the fallacy. On the other hand, experience with many repeated assays has always indicated that the probable error as shown is a very reasonable one, even though it was not obtained by the conventional procedure.

Factors Influencing Response of Rats to Vitamin D

It has already been shown that sex and color are not determinants of the degree of healing produced by a given dosage of vitamin D. Weight, gain in weight, and food consumption remain to be investigated. The 200 rats in the probable error experiment ranged in weight from 44 to 100 gm. when put on test, the average weight being 66.2 gm. The average weight of 95 rats showing 2+ healing or less (mean, 1.2+) was 65.6 gm., and of 105 rats showing more than 2+ healing (mean 3.3+) was 66.8 gm. Although no correlation was evident in this tabulation, the data were re-examined by grouping the protocols into definite weight ranges, and comparing the corresponding healing scores. It was again evident that, for the range studied, *the weight of the rat does not appreciably influence the degree of healing.*

The individual gains in weight during the 5 day test period ranged from 0 to 11 gm., the average gain being 3.0 gm. The average gain of the rats showing 2+ healing or less was 2.8 gm., and of the rats showing more than 2+ healing, 3.3 gm. This difference would seem more than accidental, but its significance is not entirely clear. Probably it reflects the fact that the rats which showed greatest healing ate the most food during the 1st day (see below).

The individual daily food consumption over the 5 day test period ranged from 4.6 gm. to 9.6 gm., the average being 6.5 gm. The average daily consumption of the rats showing 2+ healing or less was 6.2 gm., and of the rats showing more than 2+ healing, 6.7 gm. Here again the difference probably reflects nothing more than the fact that the rats which showed greatest healing were those which ate the most food during the 1st day.

The food consumption during the 1st day of the 5 day test

ranged from 2 gm. (the minimum allowable) to 12 gm. The latter figure probably indicates some scattering of food. The average was 6.9 gm. The protocols were grouped, gm. by gm., over this range, the corresponding healing scores being noted. Only eight rats ate less than 4 gm., and only seven rats ate more than 10 gm. during the 1st day. The extremes may be disregarded, therefore, as involving an insufficient number of cases. Now, if we consider the 4 gm. to 10 gm. groups inclusive, we find a definite correlation between food consumed during the 1st day and healing

TABLE I

Showing the Relation of 1st Day Food Consumption to Healing Observed by the Line Test

1st day food consumption	No. of rats	Line test findings	
		Average healing grade	Indicated cod liver oil coefficient
gm.			
2	4	*	*
3	4	*	*
4	13	1.9+	95×
5	22	2.0+	100×
6	39	2.1+	104×
7	45	2.3+	115×
8	41	2.6+	136×
9	15	2.6+	136×
10	10	2.8+	153×
11	4	*	*
12	3	*	*

* Insufficient number of animals to give significant values.

observed (Table I). The relation could be indicated graphically by an almost straight line.

The data in Table I are important in evaluating the two methods most used for administering vitamin D to test animals. McCollum's original procedure (which we follow) consists in adding the vitamin solution to the rickets-producing ration on a percentage basis. This is justified by the hypothesis that the more food the rat eats, the more vitamin should be available to compensate the excess calcium. The other-method, extensively used in Europe, consists in administering the vitamin in fixed doses by mouth.

This is justified by the hypothesis that the Ca:P ratio is more important in rickets than the absolute quantities or excesses of calcium.

An analysis of Table I indicates that the truth falls between the two hypotheses. We see that an assay performed with rats consuming 10 gm. of food during the 1st day would indicate a potency of 153 × (by Fig. 4), as against 95 × for rats consuming 4 gm. Now actually the rats consuming 10 gm. ingested 2.5 times as much vitamin D as those consuming 4 gm. Nevertheless, they gave evidence of only $\frac{153}{95}$ or 1.6 times as much. Thus it appears that rickets is influenced by both the absolute quantity of Ca and P ingested, and the ratio of Ca to P. It is apparent that so far as the accuracy of the line test is concerned, it makes little difference whether the vitamin is administered mixed with diet or separately by mouth, since the errors of the two procedures are of approximately the same magnitude, though opposite.

For work requiring the greatest possible accuracy a correction factor, derived by graphic analysis of Table I, may be applied to the potency calculated for the substance assayed: (1) Add together the 1st day food consumptions of the rats used in the assay. (2) Divide the total by the number of rats employed. (3) Calculate the difference between this quotient and 6.9 gm. (4) Decrease the calculated potency by 8 per cent for each gm. of positive difference, or increase it 8 per cent for each gm. of negative difference. In all assays to which this correction has been applied, the calculated probable error is decreased by one-eighth, regardless of the magnitude of the correction.

Relation of Curative Period to Curative Dose

The fact that the average daily food consumption bears little relation to the healing produced, whereas the 1st day food consumption exerts a marked effect, suggests that 4 or 5 days are required for the vitamin to initiate the deposition of calcium salts in the bone. This is in keeping with the fact, discovered by one of us (C. E. B.) in 1923, that 5 days is the minimum curative period for a satisfactory line test assay. We observed long ago that less vitamin D is required when the curative period is lengthened.

Different workers employ different curative periods, 5, 7, 10, 14,

and 21 days being common examples. This is particularly unfortunate when results from several laboratories must be compared, and nothing exact is known of the relation of time to curative dosage. We have now determined the time-dosage-healing relation by means of assays on activated ergosterol performed in accordance with the precision technique described above.

From a very potent stock solution a series of dilutions was made so as to give, on the basis of preliminary assays, test preparations
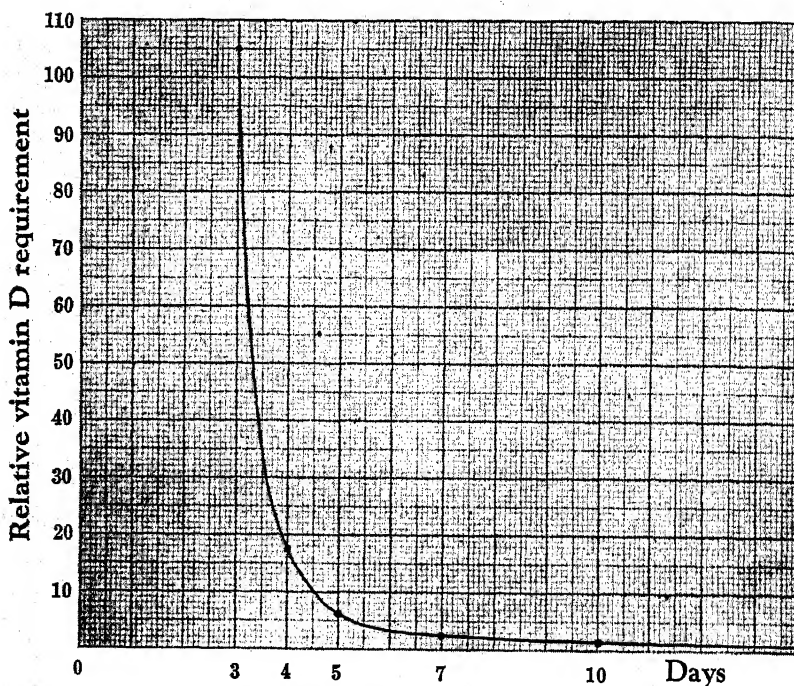
FIG. 6. Relation of dosage required to duration of test period

inducing 2+ healing when fed at ¼ per cent for different periods of time. Then large numbers of rats were given these preparations for 3, 4, 5, 7, 10, and 14 days. The exact relative dosage requirements for 2.0+ healing were computed by graphic analysis. Since our system of calculation from graphs was worked out for the standard 5 day test period, we feared that errors might be introduced in applying the graphs to periods other than 5 days. Consequently the preliminary tests were carried out with a sufficient

number of animals, so that the necessary 2+ dilution was closely approximated, and only slight correction was required from the graphs.

The results are illustrated in Fig. 6. The graph involved 95 rats; its probable error is therefore about 4 per cent. Taking the dose required for 2.0+ healing in 14 days as 1, the requirements for other periods are indicated on the vertical axis. Thus we see that 1.36 doses are required for 10 days, 2.67 for 7 days, 6.12 for 5 days, 17.6 for 4 days, and 105.1 for 3 days. The asymptotic nature of the curve is interesting, though not unexpected. The position of the 5 day test is particularly significant, for it shows that shorter test periods are not sufficiently sensitive for the detection of vitamin D in products of feeble potency, and that longer periods are unnecessary.

Recent work from our laboratory indicates (4) that the vitamin D of irradiated ergosterol and that of cod liver oil are not identical, for chickens required 100 times more of the former than of the latter, in the form of solutions equipotent for rats. It occurred to us that additional evidence of dissimilarity between the synthetic and natural forms might be found in a difference in the *rate* of healing for one species. We have seen that 4.5 times as much irradiated ergosterol was needed to produce 2.0+ healing in 5 days as in 10 days. A similar comparison made with cod liver oil showed that 4.6 times as much was needed in 5 days as in 10 days. This slight difference is well within the probable error. Thus it is evident that the time-dosage curve applies equally well to irradiated ergosterol and cod liver oil.

<center>DISCUSSION</center>

The graphic method of interpreting line tests and estimating their probable error is no substitute for care in conducting assays. Many, perhaps most, of the factors affecting calcification remain unknown. About one-fourth of the rats used for 5 day assays have to be discarded because of failure to grow or eat. We once thought that this trouble might be eliminated by the use of a better diet, but an investigation of rickets-producing rations, too extensive to be reported here, has convinced us that to the extent which a ration supports better growth than Diet 3143, it produces poorer rickets.

The number of animals which fail to grow can be reduced by lengthening the test period. Thus animals which lose weight and commence to recalcify in 5 days have an opportunity to resume growth. To what extent such animals increase the error of long period assays is not known.

We have refrained from expressing potency in terms of units per quantity of material. Such expressions are necessarily involved in cumbersome definitions, the misunderstanding of which is strikingly evident in current medical literature. While the cod liver oil coefficient expresses potency as accurately as assays can be conducted, most of the units endow assays with big figures that are not significant. This is illustrated by a tabulation which we made for comparing reports from different laboratories. An antiricketic agent having a cod liver oil coefficient of 100 (otherwise designated 100 ×) shows by the

German (Holtz) system..............	15 clinical units per cc.
" " "	1,500 rat units per cc.
New and Nonofficial Remedies (Steenbock) system.....................	300 D potency.
" "	4,000 rat units per gm.
British official (1930) system........	10,000 " " " cc.
Oslo (Poulsson) system..............	12,000 " " " gm.
Patch (Holmes) "	26,500 " " " "

SUMMARY

A critical study has been made of the line test, with special reference to technique, graphic calculation of the cod liver oil coefficient, factors influencing calcification, the dosage-time-healing relation, and the probable error.

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THE NON-VOLATILE ORGANIC ACIDS OF GREEN TOBACCO LEAVES*

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Accurate information on the organic acids of leaves is surprisingly meager; even the identity of the acidic substances in many of the plant tissues that have been investigated is still in doubt. Franzen and Keyssner (1) pointed out some years ago that the identity of the malic acid had been conclusively proved in only 15 of 235 plants reported in the literature to contain this substance and a series of critical reviews by Franzen and his associates (2–4) showed that the available information for the distribution of citric, tartaric, succinic, and lactic acids is almost equally unsatisfactory. Franzen's own extensive studies of the acids of fruits and leaves (*e.g.* (5, 6)) demonstrated the nature of the predominating acids as well as the identity of the accompanying acids but permitted statements only of the relative proportions of these substances, not of their absolute amounts, in the tissue.

Malic, citric, tartaric, and lactic acids are probably the most widely distributed of the aliphatic organic acids commonly found in aqueous extracts from green leaves. Oxalic and succinic acids are also frequently found; fumaric, malonic, and glycolic acids have been occasionally observed. The function of these substances in the metabolism of the leaf is unknown. They obviously play some part in the stabilization of the reaction of the cell as most of them have considerable buffering capacity at hydrogen ion activities within the range usually observed in leaf extracts, but opinions differ as to whether they are metabolites

of carbohydrates or of protein. Many recent workers, however, appear to incline to the latter view (7, 8).

The strongly basic alkaloid nicotine occurs in the leaves of the tobacco plant in relatively high concentration, frequently more than 3 per cent of the dry weight. The reaction of extracts from these leaves is, nevertheless, almost invariably faintly acid (about pH 5.5). A study of the acidic substances present is therefore a prerequisite to the understanding of the acid-base relationships in the cells. Garner, Bacon, and Foubert (9) have obtained data on the oxalic, malic, and citric acid content of samples of dried green leaves and partially cured leaves of several varieties of Connecticut tobacco. They employed an indirect method proposed by Kissling (10).[1] Cured and fermented tobaccos of oriental types have been extensively investigated by the Russian workers at Krasnodar, and a summary of their results was recently given by Schmuck (11). Fumaric, oxalic, malic, and citric acids were isolated and identified positively in the course of their qualitative investigations; the identification of succinic and caffeic acids was less satisfactory. Malic, citric, and oxalic acids were likewise identified in green leaves (12).

Although malic, citric, and oxalic acids appear to be the predominating acids of tobacco leaves, the other acids being present

[1] Kissling mixed 10 gm. of finely ground tobacco with an equal weight of 20 per cent sulfuric acid and a small quantity of pumice, extracted the mixture with ether for 20 hours, and dissolved the ether extract in water. Half of this solution was treated with calcium acetate in the usual way for the determination of oxalic acid. The other half was neutralized with barium hydroxide, and alcohol was added to make 20 per cent by volume. The precipitate was filtered off at once and the alcohol concentration of the filtrate was raised to 70 per cent. The precipitate so produced was filtered after several hours. The two precipitates were ignited and the small amount of barium oxide formed was converted to carbonate by the addition of a little ammonium carbonate solution followed by gentle ignition. The weights of the ashes, assumed to be barium carbonate, were taken as the equivalents of the sum of the oxalic and citric acids for the first precipitate and as the equivalent of the malic acid for the second. This method, although it gave fairly satisfactory results on mixtures of the three pure acids, was not regarded by Kissling himself as very accurate. He recognized the inadequacy of the separation of the acids and also the difficulty of securing a quantitative extraction by ether. Furthermore it may be pointed out that no account is taken of the possibility that other substances may contaminate the precipitates.

only in small amounts, the value of the quantitative determinations of these three substances that have been published is dubious, owing to the universal use of indirect methods of analysis. Our investigations of the acids of green tobacco leaves have been conducted with the view of obtaining information not only of the nature but also of the quantities of these substances present. To this end we have subjected the various methods of organic acid analysis that have been proposed to critical study. The only method that promised to give trustworthy qualitative and quantitative results is that employed by Franzen and his associates (13) in the investigation of a variety of vegetable tissues. We have devised modifications of this method by which results of approximately quantitative significance may be secured. It is the purpose of this paper to describe these modifications and to show their application to the determination of the organic acids in the tobacco plant at various stages of growth.

Lead salts have been used by practically all investigators since Scheele (14) as reagents to precipitate the organic acids from plant extracts, basic lead acetate being probably most extensively employed although it has many disadvantages. This reagent is by no means highly selective; much carbohydrate material is found in the precipitates produced by it and Vickery and Vinson (15) found that it precipitates considerable proportions of widely diversified nitrogenous substances from extracts of alfalfa leaves. The lead salt of succinic acid (16) redissolves if an excess of basic lead acetate is added, and the directions in the literature for the use of this reagent contain many empirical prescriptions on the adjustment of the reaction and on other points that suggest that much difficulty has been experienced with it.

Some years ago one of us observed (17) that barium hydroxide, when added in excess to extracts from alfalfa leaves or from yeast, on the further addition of alcohol, yielded precipitates that contained much non-nitrogenous organic material. Subsequent investigation (unpublished) revealed the presence of large amounts of organic acids but of very little nitrogen in these precipitates. We have therefore studied the effect of this procedure[2] on pure

[2] It is interesting to recall that Scheele discovered malic acid as a result of adding alcohol to an apple extract that had been neutralized by boiling with calcium carbonate.

organic acids as well as upon extracts from green tobacco leaves. It was found that a mixture of equal amounts of 1 per cent solutions of citric, oxalic, malic, succinic, maleic, and tartaric acids, when treated with excess of barium hydroxide and 2 volumes of alcohol, yielded a precipitate that could be extensively washed with 60 per cent alcohol without passing into solution. After decomposition of the precipitate with sulfuric acid, a solution was secured that contained 99.5 to 101.5 per cent of the original acidity due to organic acid as indicated by titration according to Van Slyke and Palmer (18).

That malic and citric acids are practically completely precipitated as their barium salts from tobacco leaf extracts by the addition of alcohol was shown by an investigation of the barium salts precipitate and of the filtrate from it. The precipitate was decomposed by sulfuric acid and the organic acids were extracted from the solution in a continuous ether extraction apparatus. The filtrate was likewise acidified and extracted with ether. Only about 77 per cent of the total ether-extractable acidity was found in the barium salts precipitate, but the filtrate contained no malic nor citric acids nor other acids that form silver salts insoluble at pH 7.0. To make certain that the malic acid had indeed been completely precipitated a study was made of the distribution of malic acid in the different precipitates and filtrates obtained in the large scale operations (see below). In the absence of tartaric acid, malic acid can be determined by measurements of the optical activity of the acids extracted by ether (19). Careful investigation of the fractions in which tartaric acid should occur showed that this acid was not present in tobacco leaves, and, consequently the polarimetric method for the estimation of malic acid could be applied. It was found that about 98 per cent of the malic acid of the leaf extract was precipitated by barium hydroxide and alcohol. As will later appear, citric acid is also completely precipitated from tobacco extracts by these reagents. Inasmuch as these two acids make up nearly 90 per cent of the organic acids of green tobacco leaves, the esters of which can be distilled, and also because of the completeness with which a wide variety of pure acids was precipitated in the preliminary tests, it seemed safe to assume that most of the esterifiable acids of familiar types that occur in tobacco leaf extract could be precipitated as their barium

salts from dilute alcohol. Precipitation in this way therefore appeared to offer an advantageous quantitative method to remove the simpler organic acids from extracts of tobacco leaves.

The significant features of the procedure employed can be most readily presented in diagrammatic form. Minor details, such as the washing of the precipitates, are omitted. The extract was prepared by plunging leaves, immediately after picking, into boiling water and cooking until the midribs were soft. The leaves were then removed, enveloped in canvas, and pressed at the hydraulic press. The press-cakes were ground in a meat grinder and re-extracted with boiling water twice successively. The extracts were collected quantitatively and concentrated *in vacuo*. Much inorganic material was removed by the two successive alcohol precipitations which were conducted at pH 2 so as to avoid the precipitation of calcium salts of organic acids. These precipitates were washed free from nicotine with 60 per cent alcohol.

The precipitated barium salts were centrifuged and washed once with 60 per cent alcohol and were then decomposed with 50 per cent sulfuric acid. The barium sulfate was extensively washed by decantation with hot water and was finally centrifuged. The addition of ammonia to the solution obtained by decomposing the first barium salts precipitate was designed to remove phosphoric acid. The filtrate from the precipitate so produced contained only traces of phosphate.

Filtrates A and B were separately investigated for malic acid. None could be detected in Filtrate A but 4.4 gm. were recovered from Filtrate B. The main organic acid solution contained 190 gm. of malic acid; the precipitation of malic acid was therefore carried out with a loss of not more than 2.3 per cent. The organic acids were extracted by ether in a large scale continuous extraction apparatus of the Hagemann type (20) over a period of 320 hours. The ether extract was freed from ether and from water by repeated concentration *in vacuo* after the addition of absolute alcohol and the acids were esterified as described by Phelps and Phelps (21), the distillation with alcohol vapor being continued until alcohol of a constant specific gravity came over. The esters were dissolved in ether and the dark red solution was treated with the minimal amount of 30 per cent sodium hydroxide solution

Fresh leaf
57.27 kilos = 6.65 kilos dry weight

Water extract 5 liters; acidified   Press-cake
to pH 2.0 + 1 volume alcohol

Filtrate   Precipitate 1
Concentrated to 4 liters; acidified   Gums, pectins, inorganic salts;
to pH 2.0 + 2 volumes alcohol   white granular material, weight
                                            637 gm.; N = 0.49 per cent; ash
                                            = 42.8 per cent. $[\alpha]_D^{20} = +19.5°$
                                            corrected for ash

Filtrate + excess hot satu-   Precipitate 2
rated Ba(OH)₂ solution   Weight 298 gm.; N = 0.18
                                            per cent, ash = 92.8 per cent

Filtrate A   Barium Salts 1
Ether-soluble acids = 3500 cc. 0.2 N   + H₂SO₄ to pH 1
HCl. No malic, citric, nor acid that
forms Ag salt insoluble at pH 7

Organic acids + NH₄OH   BaSO₄
to pH 8.0–9.0

Organic acids + 2 volumes   Precipitate 3
alcohol + excess Ba(OH)₂   Chiefly alkaline earth
                                        phosphates

Filtrate B   Barium Salts 2
Ether-soluble acids = 2640 cc. 0.2 N   + H₂SO₄ to pH 1
HCl. Contained 4.4 gm. malic acid

Organic acids   BaSO₄
Solids = 465 gm., N = 10.0 gm., total acid = 27,550 cc.
0.2 N HCl, ether-soluble acid = 21,250 cc. 0.2 N HCl
Extracted with ether

Ether-soluble acids, Fraction A
Total acid = 20,400 cc. 0.2 N HCl
Esterified

Ether-insoluble, Fraction B
Total acid = 6300 cc. 0.2 N HCl

Acids insoluble in 90 per cent alcohol (Fraction I)
Solids = 119 gm. = 25.6 per cent of total solids; total acid = 1400 cc. 0.2 N HCl; N = 5.0 gm.

Filtrate (Fraction J)
Carbohydrate, etc.

Esters insoluble in alkali (Fraction C)
Weight 304 gm. = 207 gm. free acid; total acid after saponification = 15,000 cc. 0.2 N HCl; subjected to fractional distillation; esters identified = 272 gm. = 89.5 per cent

Soluble in alkali (Fraction D)
Phenolic substances; acidified

Ether-soluble (Fraction E)
Phenols and phenolic acids; weight = 19.3 gm. = 4.15 per cent total solids

Ether-insoluble (Fraction F)
Weight = 53 gm. = 11.4 per cent total solids

required to neutralize the acidity. The alkaline sludge that separated was washed repeatedly with ether and removed.

The main ester fraction so obtained was washed with water until neutral and dried over anhydrous sodium sulfate. It was then freed from ether and the esters were transferred to a 500 cc. 3-necked Pyrex flask, to the central neck of which a series of fractionating columns could be fitted. The smaller necks of the flask carried capillary tubes to admit a slow current of air during the vacuum distillation. The distillation was begun with a small column of the type described by Cooper and Fasce (22). After the low boiling esters (Fraction 1) had distilled, air was admitted and a larger bore column without dephlegmator was substituted. The apparatus was then evacuated and the malic ester was distilled. A small fraction intermediate between malic and citric esters was collected separately and the distillation was continued

until the boiling point of citric ester had been passed.   The fractionating column was then removed from the apparatus and the distillation was completed without a column.   The details of the fractionation are given in Table I.

Fraction 1 yielded no malic dihydrazide nor oxalic dihydrazide. The small amount of hydrazide secured was obviously a mixture. The esters remaining after these tests were therefore saponified by boiling with an excess of sodium hydroxide. Sodium was removed by acidifying the solution to Congo red with sulfuric acid and adding 2 to 3 volumes of absolute alcohol.   After filtering

TABLE I

Distillation of Esters of Organic Acids from Green Tobacco Leaves

Total weight of esters 310 gm.; pressure 7 to 8 mm.

Fraction No.	Boiling point	Weight of fraction	Composition
	°C.	gm.	
1	53–63	12.4	No malic nor oxalic acid dihydrazide
2	88–90	52.2	Pure malic ester
3	92–96	200.0	" " "
3 a	103–112	6.4	60 per cent malic, 40 per cent citric
4	138–140	9.4	43.6 " " citric, 56.4 " " unknown
5	142–157	4.7	16 " " " 84 " " "
6	165–175	9.5	3.14 " " " 96.86 " " "
7, 8	176–178	4.4	1.38 " " " 98.62 " " "
Residue		4.9	
Distillate and residue......		303.9 = 98 per cent recovery	

off the sodium sulfate the alcohol was distilled off and the excess of sulfuric acid was exactly removed with barium hydroxide. Fractional crystallization of the free acids gave the equivalents of 0.65 gm. of fumaric acid and 0.65 gm. of succinic acid.   The fumaric acid melted at 283–284° in a sealed tube and, when mixed with authentic fumaric acid, showed no depression of the melting point.   The succinic acid melted at 185–186° and likewise showed no depression of the melting point when mixed with pure acid. Its *o*-toluidene derivative melted at 254–255° and showed no depression of melting point when mixed with authentic material. The mother liquors of the succinic acid weighed slightly more than

6.5 gm. (corrected) when evaporated to a sirup and were optically inactive in the presence of uranium acetate. Succinic acid was undoubtedly present but none could be brought to crystallization; oxalic acid could not be detected by testing with calcium acetate. This fraction therefore contained about 8 per cent of fumaric acid, and at least 8 per cent of succinic acid; malic and oxalic acids were absent; the remaining acids have not yet been identified.

Fractions 2 and 3 were essentially pure l-malic ester. The dihydrazide from Fraction 2 melted at 179–180° and that from Fraction 3 at 178.5–179.5°; neither showed depression of melting point when mixed with pure material. The specific rotation of Fraction 2 was $[\alpha]_D^{20} = -10.34°$, and that of Fraction 3 was $[\alpha]_D^{20} = -10.88°$. This value is slightly greater than that found by Frankland and Wharton (23) ($-10.44°$) but was substantiated by observations on samples of pure malic ester secured from other samples of leaves. The free malic acid secured by saponification of a sample of the ester melted at 100–100.7° and had a neutralization equivalent of 68 (theory 67.1).

Fraction 3a was a mixture of malic and citric esters. The citric acid was determined by the pentabromoacetone method (24), the malic acid by optical rotation. Malic acid dihydrazide of melting point 179° was also isolated.

Fractions 4 to 8 did not yield solid hydrazides. The citric acid content of each fraction was determined by the pentabromoacetone method and is given in Table I. Fractions 4, 5, and 6 were united, saponified, and the free acids were subjected to fractional crystallization. The equivalent of 3.16 gm. of an insoluble acid was obtained. Fractions 7 and 8 yielded the same acid in an amount equivalent to 0.56 gm. This acid melted at 283–284° in a sealed tube and contained 41.37 per cent of carbon and 3.67 per cent of hydrogen; when mixed with pure fumaric acid the melting point was not changed. Inasmuch as fumaric acid contains 41.37 per cent of carbon and 3.45 per cent of hydrogen, there can be no doubt of the identity of this substance.

The presence of fumaric acid in the ester fractions of high boiling point can be accounted for by the assumption of partial esterification. Fumaric acid is known to be difficult to esterify completely and Anschütz and Drugman (25) found that the monoethyl

ester is a solid of melting point 66° which distils at 147° at 16 mm. pressure. Fumaric monoethyl ester should therefore, if present, distil in the fraction that contains citric ester and it is interesting to note that most of the fumaric acid found in our experiments was obtained from the high boiling fractions. The identity of the other acids in these upper fractions has not yet been ascertained. None of the ester fractions contained acids that reduce alkaline solutions of silver nitrate, and Fenton's (26) test for tartaric acid could not be obtained on the acids from the esters of high boiling point. Furthermore these esters contained only traces of optically active substances.

To recapitulate, the 57.27 kilos of fresh tobacco leaves yielded 304 gm. of distilled esters of organic acids of which 85 per cent was diethyl *l*-malate, 2.5 per cent was triethyl citrate, 1.6 per cent was mono- and diethyl fumarate, and 0.2 per cent was diethyl succinate. The remaining 10.7 per cent of the esters has not been identified but oxalic, malonic, tartaric, and isocitric acids were absent. Nearly three-quarters of the unidentified portion consisted of esters of high boiling point.

No oxalic acid was found in the hot water extract of the leaves. The residues of extracted leaves were therefore thoroughly exhausted with warm 2 N sulfuric acid and the acid extract was in turn subjected to ether extraction. Only 1.27 gm. of oxalic acid were found, the quantity being ascertained by titration with permanganate and the identity established by means of the crystalline form of the calcium salt.[3] Neither malic nor citric acid could be detected. Oxalic acid was therefore present in our specimens of leaves exclusively as an insoluble salt, probably calcium oxalate; the other acids were present in soluble form.

The results of this analysis, calculated as percentages of the dry weight of the leaves and also as percentages of the total acid obtained from the esters are given in Table II. These data are founded upon a procedure that was conducted with quantitative rigor and the identity of the several acids has been established by

[3] A specimen of calcium oxalate secured in the same way from young tobacco leaves (Table IV, third column) contained 27.5 per cent of calcium (theory 27.43 per cent). The free oxalic acid secured from it melted at 101° and this melting point was not changed by admixture with authentic oxalic acid.

means of characteristic derivatives. The examination of the press-cakes demonstrated that all of the esterifiable acids except oxalic had been quantitatively removed by the hot water extraction of the leaves and the study of the filtrates from the barium salts precipitates showed that all save insignificant amounts of malic acid had been precipitated.

The over-all recovery of the malic and citric acids was of the order of 95 per cent. Analysis of the main solution of the organic acids before ether extraction revealed the presence of 190 gm. of

TABLE II

Organic Acids of Mature Green Tobacco Leaves Which Yielded Esters That Could Be Distilled

	Dry weight	Isolated acids
	per cent	per cent
Oxalic (anhydrous).................................	0.019	0.6
Succinic..	0.006	0.2
Fumaric*...	0.05	1.6
l-Malic...	2.72	85.5
Citric (anhydrous)..................................	0.082	2.6
Unknown acids† low boiling esters calculated as fumaric...	0.114	3.5
Unknown acids high boiling esters calculated as citric..	0.190	6.0

* Anschütz and Reitler (27) observed the formation of small amounts of fumaric acid during the esterification of malic acid. It is therefore possible that the fumaric acid we have found may have been an artifact.

† Contains some succinic acid that failed to crystallize.

malic acid (polarimetric method) and 4.0 gm. of citric acid (pentabromoacetone method). The malic acid in the distilled esters was equivalent to 181 gm. and the citric acid to 5.24 gm. The procedure is therefore esssentially quantitative with respect to the predominating acids of this leaf. Nothing is yet known of the efficiency of the recovery with respect to the acids in the high boiling ester fraction, but the large scale ether extraction apparatus we employed recovered 96 per cent of the total ether-extractable acidity as compared with results obtained on a small aliquot part of the main solution in a small extractor. It is evident, therefore, that the losses even of these acids could not have been serious.

The barium salts precipitate secured from the water extract of green tobacco leaves contained 465 gm. of organic solids. Of this only 207 gm. or 44.5 per cent could be accounted for as non-volatile aliphatic organic acids. The remaining 55.5 per cent consisted of substances that formed barium salts insoluble in dilute alcohol. These, on decomposition, yielded solutions that contained material quantities of organic acids according to the results of titration by the Van Slyke and Palmer method. Of the original acidity of the barium salts fraction (27,550 cc. of 0.2 N acid) the equivalent of only 54.4 per cent (15,000 cc.) was present in the fraction that contained the ether-soluble, alkali-insoluble esters. It is clear, therefore, that only about half the organic acids in the leaves of tobacco are substances of the type usually referred to as plant acids. The chemical nature of the other half of the organic acids is obscure.

The results of attempts to fractionate the various types of acidic substances in the barium salts precipitate are shown in the diagram. About 23 per cent of the total acidity of this fraction was not extracted by ether and remained in Fraction B. Less than one-quarter of this acidity could be precipitated by 90 per cent alcohol and the soluble part contained substances that reduced copper and yielded a mixture of osazones. Substances of carbohydrate nature were undoubtedly present but no definite crystalline derivative could be isolated. The alcohol precipitate did not reduce Benedict's solution and was optically inactive. Its aqueous solution was acid to Congo red and gave an intense green color with ferric chloride.

The alkali-soluble part of the ether-soluble esters (Fraction D) contained 29 per cent of the total esters. This material, after acidification and extraction with ether, yielded 19.3 gm. of an ether-soluble dark red oil (Fraction E) that was acid to Congo red and gave an intense green color with ferric chloride which turned to violet on the addition of alkali. This color test is given by caffeic acid (3,4-dihydroxycinnamic acid). The aqueous solution of the oil reduced silver nitrate in the cold and yielded a flocculent yellow precipitate on the addition of bromine. There seems little doubt that phenolic acids were present. Fraction F, when freed from reagents, yielded 53 gm. of a pale yellow, strongly acid oil which was optically inactive and gave no color reaction with

ferric chloride. This fraction represents ether-soluble material which either failed to esterify or was non-esterifiable. A summary of the data on these different fractions of the acids of green tobacco leaves is shown in Table III.

Little has yet been learned of the solubility of the barium salts of these unknown substances but it is probable that much of the titratable acidity that remained in the different filtrates shown in the diagram was due to their incomplete precipitation. The classification of these acids must await further investigation. It is obvious that they play an important part in the acid-base

TABLE III

Organic Acids of Green Tobacco Leaves That Are Precipitated as Barium Salts by Alcohol

Solids of barium salts precipitate from extract of 57.27 kilos of leaves = 465 gm.

	gm.	per cent	
Acids extracted by ether			
Organic acids from esters, identified......	186.0	40.0	44.5
" " " " not identified.	21.0	4.5	
Phenolic acids, Fraction E...............	19.3	4.2	15.6
Non-phenolic acids, Fraction F..........	53.0	11.4	
Acids not extracted by ether			
Insoluble in 90 per cent alcohol, Fraction I................................	119.0	25.6	40.0
Soluble in 90 per cent alcohol, Fraction J*................................	66.7	14.4	

* This fraction contained other substances in addition to organic acids. Some of these are related to the carbohydrates; nitrogenous substances were also present.

equilibria of the cells but until their chemical nature has been established their relationships can only be a matter of conjecture. The presence of these substances shows, however, that indirect precipitation methods for the determination of oxalic, citric, and malic acids, such as that of Kissling, can hardly be expected to yield accurate results.

The figures we have obtained for the proportions of the several acids in tobacco leaves are much lower than those secured by Garner, Bacon, and Foubert on Connecticut Havana seed tobacco by means of the Kissling method and than those of the Russian

investigators who employed Schmuck's (11) modification of an indirect method devised many years ago by Fleischer (28). Garner, Bacon, and Foubert's results indicated the presence in the dried green leaves of Havana seed tobacco of 1.47 per cent oxalic acid, 5.24 per cent citric acid, and 10.33 per cent of malic acid. Figures of nearly as great an order of magnitude have been given by Schmuck and also by Kissling for certain specimens of cured tobacco. Comparison of these data with ours is meaningless since both the Kissling and the Fleischer methods are indirect. Although these two methods may give approximately

TABLE IV

Organic Acids of Connecticut Shade-Grown Tobacco

The figures are in per cent of the dry weight.

Acid	Seed	Young leaf	Mature leaf	Cured leaf
	per cent	*per cent*	*per cent*	*per cent*
Oxalic (anhydrous)........................	0.012*	0.078	0.019	0.06
Succinic..................................			0.01	
Fumaric..................................	+	+	0.057	+
l-Malic..................................	0.010	2.76	2.72	2.11
Citric (anhydrous)........................	0.102	0.31	0.082	0.47
Unknown acids† low boiling esters.........	0.012	0.103	0.103	0.174
" " ‡ high " "	0.013	0.059	0.190	0.25
Total acids.............................	0.149	3.31	3.18	3.06

* This figure is low as the press-cake was not examined.

† Calculated as fumaric acid; probably contains some succinic acid.

‡ Calculated as citric acid; represents distilled esters only.

accurate results when applied to a mixture of pure acids of known composition, this is no reason for supposing that they do likewise when applied to a leaf extract in which numerous interfering substances occur. In neither method is there any attempt to control the purity of the precipitates.

The modified methods for the determination of the organic acids described above have been applied to aqueous extracts of oil-free tobacco seed, to the leaves of young and of mature plants, and to cured tobacco prepared commercially from mature leaves derived from the same crop as that here investigated. The details of these other analyses will be given elsewhere (29) but the essential data are shown in Tables IV and V. Malic acid is present in

traces in the seed but reaches a very high concentration in young plants upon which four to six leaves have developed. This high concentration is maintained up to maturity, but decreases slightly during curing. The great increase in malic acid concentration occurs long before storage of nicotine in the tissue reaches a high level. Rapid increase in nicotine content is a phenomenon of the maturing leaf, not of the young leaf (12). It would seem, therefore, that malic acid synthesis is independent of nicotine synthesis.

Citric acid is the predominating organic acid of tobacco seed. Synthesis of this acid occurs in the young plant although the process is far outstripped by the formation of malic acid. At

TABLE V

Organic Acids of Connecticut Shade-Grown Tobacco

The figures are in per cent of the total acids isolated.

Acid	Seed	Young leaf	Mature leaf	Cured leaf
	per cent	per cent	per cent	per cent
Oxalic...................................	8.1*	2.4	0.6	1.9
Succinic.................................			0.3	
Fumaric.................................	+	+	1.8	+
l-Malic.................................	6.7	83.4	85.5	69.0
Citric..................................	68.5	9.4	2.6	15.4
Unknown acids† low boiling esters.........	8.0	3.1	3.2	5.5
" " ‡ high " "	8.7	1.7	6.0	8.2

* This figure is low as the press-cake was not examined.
† Calculated as fumaric acid; probably contains some succinic acid.
‡ Calculated as citric acid; represents distilled esters only.

maturity the leaves contain a much decreased proportion of citric acid, this substance being present, in fact, in smaller proportion than the unknown acids that are found in the high boiling ester fraction. The situation is reversed, however, after curing. The citric acid concentration increases more than 5-fold, while the unknown acids increase only to a small extent. Small amounts of fumaric acid were detected in all the extracts analyzed but only in the mature leaf were results of quantitative value secured.

SUMMARY

Modifications of existing methods for the determination of the organic acids in plant tissues have been devised by which approxi-

mately quantitative isolation and positive identification of the predominating acids can be secured. These methods have been applied to the determination of the acids present in extracts of the seeds of the tobacco plant, of the leaves of young and of mature plants, and of leaves that had undergone the commercial curing process.

The most important modification consists in the initial precipitation of the organic acids as barium salts in the presence of dilute alcohol. This method is quantitative with respect to the better known organic acids and is superior in many respects, particularly in convenience, to the customary precipitation of the lead salts by basic lead acetate.

It is shown that only about half the total titratable acidity of tobacco leaf extracts consists of acids of familiar types such as malic, citric, oxalic, succinic, and fumaric acids. The rest belongs to acidic substances, part of which appear to be phenolic acids, the nature of which has not yet been determined. It is further evident that, in view of the presence of these substances, indirect methods for the determination of oxalic, citric, and malic acids, such as those of Kissling and of Fleischer do not yield trustworthy results.

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ON THE TRUE SUGAR CONTENT OF SKIN AND OF MUSCLE IN DIABETIC AND NON-DIABETIC PERSONS

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Recently Folin, Trimble, and Newman (1) in the course of an investigation of the distribution and recovery of d-glucose injected intravenously into animals observed that shortly after the sugar had been introduced the skin contained increased -quantities of reducing substances and the concentration approached that in the blood. And when a series of specimens was taken from the same anesthetized animal at regular intervals after the sugar injection it was noted that the reducing content of this tissue declined in such a manner that it always remained beneath but more or less parallel to that in the circulating fluid. In these same experiments the reducing substances in muscles were found to be elevated also but to a much smaller extent than in the skin. In that research the analytical methods employed were sufficient to measure merely the total reducing substances of the tissues examined and it was not possible to state just how much of this represented true sugar. More recently Hiller, Linder, and Van Slyke (2), Folin and Svedberg (3), Somogyi (4), and Benedict (5) by their investigations have established that, by employing only short periods of fermentation with washed yeast, the true sugar of blood or of blood filtrates can be measured and have described various procedures for accomplishing this. In the light of this advance it became of interest to know to what extent the elevation of reducing substances in skin and muscle observed by Folin, Trimble, and Newman represented real increase of carbohydrate. And since the earlier research had furnished information only with regard to experimental animals it seemed desirable that upon this occasion we should devote our attention to the situation existing in human subjects.

During the preparation of this manuscript for publication there has come to our attention the recent report of Urbach and Sicher (6) upon the sugar content of skin under normal and pathological conditions. These investigators were able to employ a micro procedure in which small cylinders of skin, weighing 100 to 150 mg., were removed from an animal or a patient with an electric cutting appliance and then the total reducing substances were measured by a modified Hagedorn-Jensen method. The results which they report for single specimens from normal and diabetic individuals are in general agreement with those which we record below. Of particular interest are some experiments of these workers in which they obtained a series of specimens from the same individual, covering the 4 hours immediately after intake of glucose. The finding that under such circumstances the total reducing substance of the skin tends to rise and then fall parallel with the sugar in the blood is in agreement with that observed by Folin, Trimble, and Newman in their experiments on animals.

<div align="center">EXPERIMENTAL</div>

Plan of Experiments

Specimens of human skin and of muscle were obtained fresh from the operating rooms of The Palmer Memorial and The New England Deaconess Hospitals. Breasts removed by radical technique, limbs and feet amputated from diabetic patients, and skin cut away during abdominal operations provided liberal quantities of material from which portions of tissue well outside of any infected area were excised. Then the sugar was extracted from weighed portions of the material and its concentration determined. A sample of blood taken near the conclusion of the operation enabled us to obtain practically simultaneous values for the sugar contents of blood, skin, and muscle under the conditions studied.

Methods

Extraction of Tissues—The specimen was obtained immediately after its amputation by one of us present in the operating room at the time. It was carried to a laboratory just outside where portions of skin were quickly dissected free from underlying fat. Usually portions of 50 to 100 gm. were obtained (though much less

was employed satisfactorily upon several occasions when the amount available was small). The specimen was weighed (to gm. only) and then plunged into an approximately equal weight of boiling water contained in a beaker. Heat was applied at once and maintained until the liquid again boiled. Ordinarily the tissue was cut into small strips and sometimes was passed through a food chopper before dropping it into the hot water. It was not observed that these variations in the treatment had any effect upon the concentration of sugar found.

While one individual cared for the specimens of skin another cut off sections of muscle (pectoralis major and gastrocnemius being used most frequently), weighed them, and likewise plunged them into boiling water. It was considered important that the interval elapsing between the amputation of the tissue and the stoppage of enzyme action by heat should be as small as possible. In our experiments this was usually 5 and never more than 10 minutes.

The specimens were then removed to our laboratory for extraction and analysis. In accomplishing this it was our custom to employ three or four successive treatments for periods of 10 to 15 minutes with portions of boiling water sufficient completely to cover the tissue. The extracts were poured off through a filter of cheese-cloth (previously washed and extracted with boiling water) upon which the bulk of the tissue was collected. Then by drawing together the upper edges of the cloth and squeezing gently it was possible to press out most of the liquid from the tissue and thus insure almost complete change of the extracting fluid at the end of each treatment. The nearly dry residue was then readily returned to the extraction beaker for further treatment. The specimens used were always cut into strips or ground up by passing through a food chopper while in the first extraction liquid (if this had not already been accomplished before they were plunged into the boiling water). Also at this time the chopped tissues were further macerated by rubbing in a mortar with about 15 gm. of washed quartz sand. Finally the combined extracts were mixed, cooled, and their total weight determined. At this stage any fat which had solidified was collected, weighed, and then an appropriate correction was applied to the weight of the tissue originally taken for analysis.

Removal of Protein—In the case of blood the procedure of Folin and Wu (7) was employed. As in our previous research the extracts of muscle and of skin were freed from protein and clarified by precipitation with tungstic acid, followed by treatment with Lloyd's alkaloidal reagent (8). In detail the procedure has been as follows: We customarily found it convenient to take 10 volumes (40 cc.) of extract and to add 1 volume (4 cc.) each of 10 per cent sodium tungstate and of $\frac{2}{3}$ N sulfuric acid. After shaking vigorously and filtering, 20 cc. of the filtrate were mixed with 5 cc. of 0.1 N sulfuric acid and about 1.5 gm. of Lloyd's alkaloidal reagents were added. This was agitated gently for 2 minutes and then filtered.

Measurement of Total Reducing Substances—This last filtrate was analyzed for total reducing substance with the modified copper reagent of Folin and Wu (9). Usually 2 cc. of this filtrate contained a quantity of reducing substance appropriate for colorimetric comparisons. At times, however, it was necessary to make dilutions of this material before analysis or to employ a more dilute sugar standard. Anhydrous glucose from the United States Bureau of Standards served for the preparation of all standard solutions. In computing the concentration of reducing substances per 100 gm. of fresh tissue the concentrations found in the filtrate analyzed were multiplied by factors which compensated for the dilutions during protein removal and treatment with Lloyd's reagent. This product when multiplied by the total volume of extract and divided by the weight of tissue taken gave the concentration of total reducing substance (expressed as mg. per cent) in the fresh specimen.

Measurement of Non-Fermentable Reducing Substance—For this we have employed fermentation by yeast by means of a procedure essentially like that of Benedict ((5) p. 465). In detail our process has been as follows:

Preparation of Washed Yeast—In each of a series of centrifuge tubes about one-sixteenth of a cake of Fleischmann's compressed yeast was placed and 10 cc. of distilled water added. With a stirring rod the yeast was rubbed and stirred until a uniform suspension resulted. Then the tubes were centrifuged and the supernatant fluid decanted. A fresh portion of distilled water was added and the washing process twice repeated. Treated in this

way the fourth aqueous extract of the yeast gave a negligible blank with the Folin-Wu copper reagent. If the material employed was not fresh, or if it had stood in a refrigerator after having been washed a day or more previously, satisfactory blanks were not obtained.

Fermentation of the Tissue Extract—10 cc. of the filtrate obtained after the treatment with Lloyd's reagent (see above) were added to a centrifuge tube containing washed yeast. The contents were thoroughly mixed until a uniform suspension resulted and then placed in a beaker of water maintained at 37–40° for 15 minutes. The mixtures were centrifuged to throw down the yeast cells. 2 cc. of the supernatant fluid were analyzed for reducing substance, by means of the revised Folin-Wu copper method. A sugar standard containing one-fifth of the glucose in the weak standard of Folin and Wu was frequently necessary. In every instance an aqueous extract of the yeast employed was analyzed at the same time and in no case was it found that the washed yeast used was contributing any appreciable portion of the non-fermentable residue found. The concentration of non-fermentable material extracted from the tissues was computed in a manner similar to that mentioned above for the total reducing substance. The values for the true sugar present in 100 gm. of blood or of tissues were calculated by subtracting the non-fermentable fraction from the total of reducing substances found before fermentation. During the course of these experiments accurately measured quantities of pure glucose in varying amounts were introduced into different extracts before precipitation of the protein. The sugar added was accounted for quantitatively by the increase of total reducing substance. Added glucose caused no change in the non-fermentable reducing material.

Results

The results obtained are presented in Table I. In the case of the non-diabetic subjects it may be observed that in every instance the total reducing substance of the blood was elevated slightly above the normal level—which is accounted for by the anesthesia employed. In a number of these cases the non-fermentable material of the blood was not measured. However, our experience with similar material indicates that this fraction usually

TABLE I

Simultaneous Measurements of Sugar Content of Human Blood, Skin, and Muscle

Reducing substances are given in mg. per cent.

Subject	Blood			Skin			Muscle			Insulin dosage	
	Total	Non-fermentable	True sugar	Total	Non-fermentable	True sugar	Total	Non-fermentable	True sugar	Units	Hrs. previously
Non-diabetic cases*											
Mrs. B-y	152†			86	17	69	78	43	35		
" P-t	136			78	20	58	63	32	31		
" T-r	124			56	10	46	64	34	30		
Miss C-y	122			76	13	63	57	32	25		
Mrs. S-n	120			64	6	58					
Mr. S-r	119			66	12	54					
" P-t	108	10	98	58	10	48	47	28	19		
Averages......	126			69	12	56	61	33	28		
Diabetic cases‡											
Mrs. Y-e	360	15	345	245	23	222	108	36	72	12	4
" W-t	340	15	325	234	23	211	104	28	76	7	17
Mr. L-n§	339			240	30	210	109	32	77	5	4
Mrs. B-a	298			226	15	211	134	33	101	0	
" I-n	295	14	284	200	18	182	79	31	48	10	3
Mr. V-t§	266	13	253	189	12	177	102	35	67	10	4
Mrs. M-d	260			188	33	155	88	48	40	10	4
Mr. C-e	228	15	213	125	12	113	70	38	32	0	
Mrs. S-s	196	12	184	111	13	98	64	26	38	5	17
Mr. L-n‖	186	16	170	121	16	105	75	29	46	10	2
" V-t‖	170	18	152	105	18	87	75	32	43	10	4
Mrs. H-k	154			128	36	91	47	27	20	8	4
Mr. R-l	129			87	9	79	71	29	42	10	5
" S-n	120	9	111	92	9	83	44	29	15	10	17
Averages......	238	14	226	163	19	144	83	33	51		

* Gas-ether anesthesia was employed in all of the non-diabetic cases.

† All values were calculated in terms of glucose.

‡ Novocaine-spinal anesthesia was used for all of the diabetic cases except nitrous oxide for Mrs. I-n and Mr. C-e.

§ First operation.

‖ Second operation.

constitutes in the neighborhood of 15 mg. per cent when the Folin-Wu copper method is used. The true sugar of the whole blood in these non-diabetic cases therefore may be estimated without serious error by subtracting this quantity from the total reducing substance reported. This would give an approximate average value of 110 mg. per cent for our cases. The total reducing substance in the skin of non-diabetic subjects averaged somewhat more than one-half of that found in the blood, and when allowance for the non-fermentable portion is made that ratio is lowered only slightly. In muscle the total of reducing substances approached that in the skin, but the proportion which was fermentable was smaller. This indicates, therefore, that the true sugar of non-diabetic muscle is considerably below both that in the skin and the blood. Had our analyses been restricted to that portion of the circulating fluid which is in most intimate contact with the tissues then the ratios obtained would be even less favorable to skin and muscle than those mentioned, since the plasma is known to contain but little of the non-fermentable reducing material.

With the diabetic individuals it is readily evident that the total reducing material of the skin was increased very definitely and decidedly above non-diabetic levels, while the non-fermentable portion was augmented but little. The data obtained from the diabetic cases are recorded with the values of the total reducing substance found in the blood arranged in a descending order. In general it will be noted that this, with few exceptions, likewise arranges the true sugar content of the skin in gradually decreasing concentrations. The true sugar of the skin appears to average about 65 to 75 per cent of that in the blood. That this relationship between blood and skin sugar likewise holds true for a given individual when observed at different times is shown by the data recorded for Mr. L-n and for Mr. V-t. In these two cases we were fortunate in being able to obtain two successive specimens at intervals several weeks apart. In each instance clinical treatment had lowered the blood sugar level and this was reflected in the tissue sugar content as well.

In the muscles of the diabetic subjects the total of reducing substances was greater than that in the non-diabetic subjects, while that fraction which was fermentable averaged approxi-

mately the same in both series. The net result indicates a slight but definite increase in the true sugar of the diabetic muscle. In the non-diabetic muscles the true sugar content averaged about one-fourth of that of the blood. With the diabetic cases the observed true sugar values did not follow the changes in the blood with as much regularity as with the skin. However the tendency to approach the relation existing between non-diabetic blood and muscle is evident.

A majority of the diabetic patients whom we have had an opportunity to study had received insulin several hours before the tissues were removed. It seemed desirable to ascertain whether this factor might have been responsible for the surprisingly low values for the true sugar of muscle obtained in such cases. Therefore we have been able to obtain a few specimens where insulin had not been administered at all, or where it had been omitted for a period sufficient for its action to have been exhausted. This group comprises about one-third of the diabetic cases recorded in Table I. The individual instances may be located by reference to the data on insulin dosage which are given there. If attention is focused upon those who had not received insulin for at least 17 hours previous to the surgical operation at which their tissues were removed it will be noted that only in one instance (Mrs. B-a) did we encounter any value for muscle sugar which could be classified as definitely higher than in those individuals with corresponding blood sugar levels where recently administered insulin could still be exerting an influence. On the other hand, if the muscle sugar values of the insulinized and non-insulinized groups are averaged and compared the values are, respectively, 51 and 52 mg. per cent. The series of observations presented is rather brief and the similarity just mentioned may be merely a coincidence. However it furnishes but little support for an attempt to ascribe the low values for muscle sugar recorded above solely to the externally administered insulin.

SUMMARY AND CONCLUSIONS

Data upon the total reducing substance, the non-fermentable material, and the true sugar content of specimens of blood, skin, and muscle from non-diabetic and from diabetic patients are presented. In the non-diabetic group the true sugar content of

skin and of muscle averaged respectively 56 and 28 mg. per cent. For some cases of diabetes the corresponding averages were 144 and 51 mg. per cent when the average true sugar of the whole blood was 226 mg. per cent.   Thus it is shown that elevation of the sugar concentration in the blood is accompanied by a marked absolute increase of the quantity in the skin while the elevation in muscle is much smaller.   The sugar content of muscles from diabetics who had received insulin at short intervals previously averaged almost the same as in the group where externally administered insulin was no longer believed to be exerting an effect.

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THE ALLEGED PRESENCE OF "BOUND POTASSIUM" IN MUSCLE

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The importance of potassium in maintaining muscle tone was considered in a publication by Neuschloss in 1923 (1). Muscles of toads and rabbits, after remaining for a short period of time in potassium-free Ringer's solution, lost their physiological tone and their ability to contract. In 1924 Neuschloss (2), published a method for determining the amount and condition of the potassium in the muscle. He related the potassium held by the muscle, when it was placed in potassium-free Ringer's solution, to muscle tone. The greater part of the potassium was found in the solution and only a small part, designated as "bound potassium," was held by the muscle. In later publications (3) Neuschloss showed variations in the amount of bound potassium in red and white muscle of rabbits, the relation of varying quantities of NaCl, KCl, and CaCl₂ to bound potassium, the effect of electrical and chemical stimulation, etc.

In 1927 Raab (4) attempted to repeat Neuschloss' method of determining bound potassium. He modified Neuschloss' method by using a smaller volume of salt solution, calculating his results on wet weight basis, and coagulating the protein during the determination. With this procedure Raab was unable to confirm Neuschloss' results and concluded (1) that the loss of potassium by muscle placed in isotonic salt solution followed the laws of diffusion; (2) that loss of function of muscle was not related to bound potassium as determined by Neuschloss' method. Neuschloss (5) criticizing Raab's work, contended that the accumulation of acids, autolytic action, and the small volume of salt solution used, were the causes of failure to confirm his results.

Raab's work was upheld by Höber (6) who showed that the small volume of salt solution did not explain the variation. The presence of acid hindered rather than aided the loss of potassium by the muscle. Stirring the solution, and varying the size of

TABLE I

Rabbit Gastrocnemii in 0.9 Per Cent Saline Solution

Experiment No.	Conditions	Initial value	Mg. K per 100 gm. dry tissue							
			1 hr.	3 hrs.	6 hrs.	12 hrs.	18 hrs.	24 hrs.	30 hrs.	48 hrs.
1	Small amount of saline	18.2	7.0	2.2	1.7	1.1	1.2			
2	30 cc. saline per gm. wet tissue	14.9	3.0 (2 hrs.)	1.3	0.51	0.70	0.59		0.67	
3	60 cc. saline per gm. wet tissue	15.5	2.2	0.64	0.48	0.51		0.35	0.31	
4	Muscle cut as usual. 300 cc. saline changed frequently	18.7	4.5		1.8	0.08	Trace	Trace	Trace	
5	Muscle cut in very small pieces. 300 cc. saline changed frequently	19.0	2.4	2.0	0.09	Trace	"	"	"	
6	30 cc. saline per gm. wet tissue at 5°	15.7	4.9	1.4	1.5	0.65		0.47		0.45
7	30 cc. saline per gm. wet tissue at 20°	12.0	3.4	2.9	0.46	0.42		0.62		0.49

muscle particles changed the amount of potassium found in the solution. Höber confirmed Raab's contention that muscle tone and bound potassium are unrelated.

In connection with studies of inorganic ion changes in muscle, our attention was called to Neuschloss' work. Attempts to

determine bound potassium by Neuschloss' procedure were unsuccessful and the following results support the contention of Raab and Höber.

The gastrocnemii muscles of rabbits were cut into small pieces and each muscle placed in isotonic salt solution (0.9 per cent). Samples of the muscles were taken at the beginning of the experiment and usually at the end of 1, 3, 6, 12, 18, and 24 hours. The samples were placed in vitreosil crucibles, and dried at 100° to constant weight. The weighed, dried tissue was treated with a small amount of 4 N sulfuric acid and ashed in a muffle furnace· at a temperature of approximately 600°. Shohl and Bennett's method (7) of determining the amount of potassium was used. Potassium values were expressed as mg. of potassium per 100 gm. of dried tissue.

The values obtained (Table I, Experiment 1) for the amount of bound potassium were higher than those given by Neuschloss (1.2 as against 0.36). On increasing the quantity of saline to 30 cc. and 60 cc. per gm. of wet muscle, the values for the bound potassium were much lower (Table I, Experiments 2 and 3) and the values varied considerably. Having secured a change in bound potassium by increasing the amount of saline, large quantities (300 cc.) with frequent changes of saline were tried (Table I, Experiment 4). On cutting the muscle into very small pieces (Table I, Experiment 5) the bound potassium values decreased more rapidly and only a trace remained in the muscle at the end of 12 hours. Such a change in the amount of potassium was not expected as Neuschloss found that regardless of the size of the particles and the amount of saline used, the values for the bound potassium remained the same after the 6th hour until autolysis began.

To decide the importance of autolysis, the gastrocnemii were cut into small pieces and each muscle placed in saline, the proportion 30 cc. of saline to 1 gm. of muscle being used. One solution containing the small pieces of one gastrocnemius was placed in the ice box and kept at a temperature of 5°. The solution containing the opposite muscle was kept at room temperature (20°). From the results (Table I, Experiments 6 and 7) it would appear

that autolysis plays little part for the first 24 hours in the potassium leaving the muscle under the experimental conditions.

SUMMARY

The results obtained by using Neuschloss' method for determining bound potassium were unsatisfactory. The decrease in bound potassium in muscle tissue placed in isotonic saline is related to the amount and frequency of change of isotonic salt solution and to the size of the pieces of muscle tissue. Autolysis for a period of 24 hours did not apparently affect the diffusion of potassium into the solution. The present experiments furnish no evidence ·for the existence of bound potassium in muscle tissue.

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THE CONFIGURATIONS OF THE SECONDARY CARBINOLS OF THE ISOPROPYL AND OF THE ISOBUTYL SERIES

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The configurations of normal aliphatic secondary carbinols can now be correlated with that of methylethyl carbinol on the basis of the direction of their rotations; namely, all dextrorotatory normal secondary carbinols have the following configuration

$$
\begin{array}{c}
R_1 \\
| \\
HCOH \\
| \\
R_2
\end{array}
$$

I
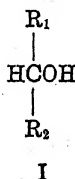
where R_1 stands for the lighter radical and R_2 for the heavier as in the cases of

$$
\begin{array}{ccc}
CH_3 & & C_3H_7 \\
| & & | \\
HCOH & or & HCOH \\
| & & | \\
C_2H_5 & & C_4H_9
\end{array}
$$
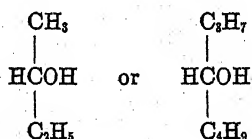
If, in the same expression, R_1 stood for the heavier and R_2 for the lighter, then the carbinol given by Formula I would be levorotatory. Thus, in the series of propyl carbinol,

$$
\begin{array}{cccccc}
C_3H_7 & C_3H_7 & C_3H_7 & C_3H_7 & C_3H_7 & \\
| & | & | & | & | & \\
HCOH & HCOH & HCOH & HCOH & HCOH & etc. \\
| & | & | & | & | & \\
CH_3 & C_2H_5 & C_3H_7 & C_4H_7 & C_5H_{11} & \\
\end{array}
$$

II III IV V VI

669
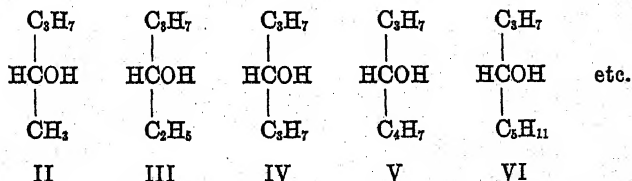
the first two members are levorotatory, the third is inactive and all the remaining members are dextrorotatory. The question arises as to the direction of rotation of the corresponding members of the series in which the normal propyl is substituted by an isopropyl group. Thus far, the configuration of only one branched chain carbinol has been correlated by direct chemical methods with that of a normal carbinol, namely, that of 2-methylheptanol-(6).[1] In this carbinol the extra methyl group is attached to the fourth carbon atom from the asymmetric carbon. The molecular rotation of this branched chain carbinol is not much different from that of the corresponding normal carbinol. It does not follow, however, that an isopropyl group located on the asymmetric carbon atom, or near to it, will not have a significant influence on the molecular rotation of the substance.

It is evident that in order to discover the effect of the isopropyl group on the rotation, it is required to correlate the configurations of the normal and the branched chain carbinols. To accomplish this by direct chemical methods is a laborious task. Work in this direction is now in progress in this laboratory. However, with a certain degree of probability, the configurations may be correlated on the basis of the numerical values of the molecular rotations of the members of the corresponding series of normal and of branched chain carbinols.

For the series of isopropyl carbinols, the molecular rotations were furnished by Pickard and Kenyon[2] and are as follows:

$$
\begin{array}{ccc}
\mathrm{CH_3} \quad \mathrm{CH_3} & \mathrm{CH_3} \quad \mathrm{CH_3} & \mathrm{CH_3} \quad \mathrm{CH_3} \\
\diagdown \diagup & \diagdown \diagup & \diagdown \diagup \\
\mathrm{CH} & \mathrm{CH} & \mathrm{CH} \\
| & | & | \\
\mathrm{HCOH} & \mathrm{HCOH} & \mathrm{HCOH} \\
| & | & | \\
\mathrm{CH_3} & \mathrm{C_2H_5} & n\text{-}\mathrm{C_3H_7}
\end{array}
$$

$[M]_D^{20}$ $+4.3°$ $+15.4°$ $+24.7°$

VII   VIII   IX

---

[1] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **83**, 177 (1929).

[2] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **101**, 620 (1912).

$$\begin{array}{cc}
\begin{array}{c}
CH_3 \quad\; CH_3 \\
\diagdown\;\diagup \\
CH \\
| \\
HCOH \\
| \\
n\text{-}C_4H_9 \\
+ 33.3° \\
\\
X
\end{array}
&
\begin{array}{c}
CH_3 \quad\; CH_3 \\
\diagdown\;\diagup \\
CH \\
| \\
HCOH \\
| \\
n\text{-}C_5H_{11} \\
+ 32.9° \\
\\
XI
\end{array}
\end{array}$$
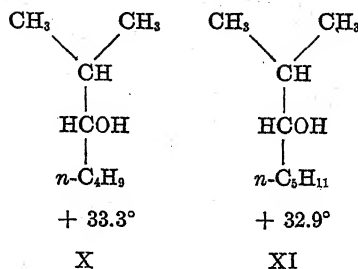
The third member of this series has a molecular rotation of $+24.7°$ whereas in the normal series this member is symmetric, hence inactive. If it is then assumed that the substitution of the propyl group by an isopropyl group introduces in every member a change in rotation approximating the value of the rotation of the isopropylpropyl carbinol, then the first and the second members (VII and VIII) which correspond to the levorotatory members of the normal series (II and III) should have values of molecular rotation lower than $+24.7°$ and the member which corresponds to the dextrorotatory member should have a higher value than $+24.7°$. On this assumption the series of isopropyl carbinols rotating in the same direction belong to one series and the dextrorotatory series has the configuration given above. Had it been assumed that the opposite configuration belonged to the dextrorotatory series, then the values of their molecular rotations should have been of descending order.

The latter condition is encountered in the case of the isobutyl series. Pickard and Kenyon[3] resolved three members but only one to completion. Five were resolved by us as far as present methods permit and their molecular rotations are given in the following set of figures.

$$\begin{array}{ccc}
\begin{array}{c}
CH_3 \quad\; CH_3 \\
\diagdown\;\diagup \\
CH \\
| \\
CH_2 \\
| \\
HOCH \\
| \\
CH_3 \\
+ 21.12°
\end{array}
&
\begin{array}{c}
CH_3 \quad\; CH_3 \\
\diagdown\;\diagup \\
CH \\
| \\
CH_2 \\
| \\
HOCH \\
| \\
C_2H_5 \\
+ 24.61°
\end{array}
&
\begin{array}{c}
CH_3 \quad\; CH_3 \\
\diagdown\;\diagup \\
CH \\
| \\
CH_2 \\
| \\
HOCH \\
| \\
n\text{-}C_3H_7 \\
+ 16.30°
\end{array}
\end{array}$$
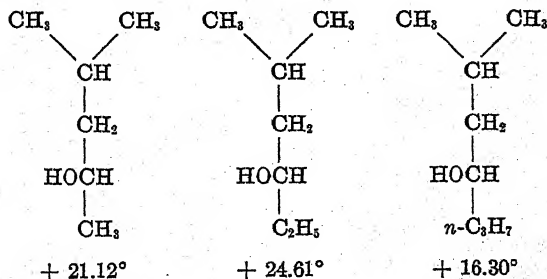
[3] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **105**, 101 (1914).

$$CH_3 \quad CH_3 \qquad CH_3 \quad CH_3$$
$$CH \qquad\qquad CH$$
$$CH_2 \qquad\qquad CH_2$$
$$HOCH \qquad\quad HOCH$$
$$n\text{-}C_4H_9 \qquad\quad n\text{-}C_5H_{11}$$
$$+ 13.76° \qquad\quad + 11.26°$$
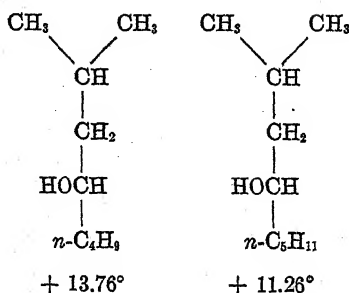
Again in this case, the isobutylbutyl carbinol has an appreciable rotation. The values of the molecular rotations, however, in this series are of descending order. Taking again the corresponding normal butyl series, we find that

$$C_4H_9 \qquad C_4H_9 \qquad C_4H_9 \qquad C_4H_9 \qquad C_4H_9$$
$$HOCH \quad HOCH \quad HOCH \quad HOCH \quad HOCH$$
$$CH_3 \qquad C_2H_5 \qquad C_3H_7 \qquad C_4H_9 \qquad C_5H_{11}$$

| Dextro | Dextro | Dextro | Symmetric | Levo |
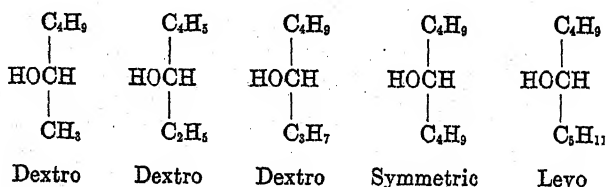
the members of the series with a descending order of molecular rotations have the configurations given above. On the assumption that the change of the propyl group to an isopropyl group has a similar effect on all members of the group, it is expected that the members preceding the isobutylbutyl carbinol should have a rotation higher than the latter and those following, a lower molecular rotation.

Thus, comparing the two series, one of the dextrorotatory isopropyl carbinols and the other of isobutyl carbinols, one notices that in one series the rotations are of the ascending order in numerical values and in the other, of descending order. On the basis of this difference, it may be concluded that the dextrorotatory carbinols of the two series are of opposite configurations. Or, in other words, isopropylpropyl carbinol and isobutylbutyl carbinol of similar configurations rotate in opposite directions. The cause of this difference is undoubtedly to be looked for in the differences in the distance of the isopropyl group from the asymmetric carbon atom in the carbinols of the two series (see Table I).

Dextro-Methylisobutyl Carbinol—The *d,l*-alcohol was prepared from isobutylmagnesium bromide and acetaldehyde. The resolution was accomplished through the half phthalic acid method of Pickard and Kenyon under conditions developed in this laboratory.

$$[\alpha]_D^{23} = \frac{+10.32° \times 100}{1 \times 18.50} = +55.8° \text{ (in absolute alcohol)}$$

The carbinol was obtained by saponifying the ester in the way described by Pickard and Kenyon.[4] B.p. 64° at 60 mm. $n_D^{25} = 1.4100$

TABLE I

Maximum Rotations in the Homogeneous State of the Series of Isobutyl Carbinols

	$[\alpha]_D^t$	$[M]_D^t$	t
	degrees	*degrees*	°*C.*
Methylisobutyl carbinol..........	20.8	21.12*	29
Ethylisobutyl "	21.23	24.61	35
n-Propylisobutyl "	12.54	16.30	33
n-Butylisobutyl "	9.48	13.76	32
n-Amylisobutyl "	7.22	11.26	30

* It is possible that this carbinol has not yet been resolved to its maximum.

$$[\alpha]_D^{29} = \frac{+16.70°}{1 \times 0.8008} = +20.85°; \ [M]_D^{29} = +21.27° \text{ (homogeneous)}$$

Pickard and Kenyon found $[\alpha]_D^{29.5} = +20.04°$.

4.225 mg. substance: 10.910 mg. CO_2 and 5.170 mg. H_2O.
$C_6H_{14}O$.   Calculated. C 70.52, H 13.81
         Found.   " 70.41, " 13.69

Dextro-Ethylisobutyl Carbinol—The *d,l*-alcohol was prepared from isobutylmagnesium bromide and propionaldehyde.

The half phthalic ester of the inactive carbinol was resolved

⁴ Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **91**, 2058 (1907).

by crystallizing its strychnine salt from acetone.   The maximum rotation of the ester obtained was

$$[\alpha]_D^{28} = \frac{+\,2.18° \times 100}{1 \times 8.80} = +\,24.8° \text{ (in absolute alcohol)}$$

The strychnine salt was decomposed with 36 per cent hydrochloric acid and the carbinol was obtained by saponification of the phthalic ester.   B.p. 81° at 60 mm. $n_D^{25} = 1.4171$.

$$[\alpha]_D^{25} = \frac{+\,17.02°}{1 \times 0.801} = +\,21.23°; \ [M]_D^{25} = +\,24.61° \text{ (homogeneous)}$$

Pickard and Kenyon[3] found $\alpha_D = 9.39°$ in a 1 dm. tube on imperfect material.

3.360 mg. substance: 8.920 mg. CO_2 and 4.215 mg. H_2O.
<div style="text-align:center">$C_7H_{16}O$.   Calculated.   C 72.35, H 13.87
Found.   " 72.06, " 13.67</div>

Dextro-n-Propylisobutyl Carbinol—The *d,l*-alcohol was prepared from isobutylmagnesium bromide and *n*-butylaldehyde. The phthalic ester of the inactive carbinol was resolved by recrystallizing its brucine salt from acetone.   The ester obtained had the following rotation.

$$[\alpha]_D^{33} = \frac{+\,1.40° \times 100}{1 \times 10.749} = +\,13.0° \text{ (in absolute alcohol)}$$

The active carbinol was then obtained by saponification of the phthalic ester.   B.p. 80° at 25 mm. $n_D^{25} = 1.4205$.

$$[\alpha]_D^{33} = \frac{+\,10.12°}{1 \times 0.807} = +\,12.54°;^3 \ [M]_D^{33} = +\,16.30° \text{ (homogeneous)}$$

Pickard and Kenyon report $\alpha_D = 4.99°$ in a 1 dm. tube.

3.625 mg. substance: 9.785 mg. CO_2 and 4.615 mg. H_2O.
<div style="text-align:center">$C_8H_{18}O$.   Calculated.   C 73.77, H 13.91
Found.   " 73.74, " 14.27</div>

Levo-n-Butylisobutyl Carbinol—The *d,l*-alcohol was prepared from isobutylmagnesium bromide and *n*-valeraldehyde.   The phthalic ester of the inactive carbinol was resolved by recrystal-

lizing its strychnine salt from acetone. The ester obtained had the following rotation.

$$[\alpha]_D^{30} = \frac{-0.60° \times 100}{1 \times 7.49} = -8.1° \text{ (in absolute alcohol)}$$

The carbinol was obtained by saponification of the phthalic ester. B.p. 87° at 20 mm. $n_D^{25} = 1.4258$.

$$[\alpha]_D^{32} = \frac{-7.62°}{1 \times 0.804} = -9.48°; [M]_D^{32} = -13.76° \text{ (homogeneous)}$$

4.575 mg. substance: 12.590 mg. CO_2 and 5.790 mg. H_2O.

$C_9H_{20}O$.   Calculated.   C 74.89, H 14.01
              Found.   " 74.98, " 14.16

Dextro-n-Amylisobutyl Carbinol—The d,l-alcohol was prepared from n-amylmagnesium bromide and isovaleraldehyde. The phthalic ester of the inactive carbinol was resolved by recrystallizing its strychnine salt from a mixture of acetone and petrolic ether. The ester obtained had the following rotation.

$$[\alpha]_D^{30} = \frac{+2.54° \times 100}{1 \times 21.27} = +11.9° \text{ (in absolute alcohol)}$$

The carbinol was obtained by saponification of the phthalic ester. B.p. 117° at 40 mm. $n_D^{25} = 1.4302$.

$$[\alpha]_D^{30} = \frac{+5.88°}{1 \times 0.814} = +7.22°; [M]_D^{30} = +11.26° \text{ (homogeneous)}$$

5.250 mg. substance: 14.620 mg. CO_2 and 6.510 mg. H_2O.

$C_{10}H_{22}O$.   Calculated.   C 75.85, H 14.03
               Found.   " 75.94, " 13.97

STUDIES ON ARGININE

I. THE RATE OF CATABOLISM OF ARGININE IN RATS, IN-CLUDING A METHOD FOR THE DETERMINATION OF ARGININE IN BIOLOGICAL MATERIAL

By VEON CARTER KIECH, JAMES MURRAY LUCK, AND ANNA EVELYN SMITH

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PART I

Determination of Arginine

Although arginine occupies a prominent position in many of the problems that engage attention in present day biochemistry, it is worthy of note that little has been accomplished in the develop-ment of analytical methods appropriate for its determination in plant and animal material. The excellent procedure developed by Van Slyke (1) and modified by Plimmer and Rosedale (2) in which arginine is converted into ornithine and ammonium carbonate by alkaline hydrolysis has been of great service in the analysis of proteins. Its lack of specificity and the size of the sample required for analysis have not permitted its use in the determination of free arginine in tissue extracts. The same difficulties apply, doubtless, to the permanganate oxidation method of Orglmeister (3), and the flavianic acid method of Kossel and Gross (4). The hypobromite method of Sakaguchi (5) lacks in specificity (6) but possesses the micro features characteristic of colorimetric tech-nique. Weber has recently applied it to the determination of arginine in blood (7). At present the methods which appear most acceptable for the estimation of arginine in such formidable material as a tissue extract, are those based upon the use of arginase. Were it not for an early observation by Dakin (8), arginase could be regarded as ideally specific in the sense that it will catalyze the hydrolysis only of *d*-arginine (9–11). It is the

most selective agent available for the estimation of this substance. Jansen (12) and Hunter and Dauphinee (13) have employed it with urease in methods in which the resulting ammonia serves as a measure of the arginine content. Finally, Bonot and Cahn (14) have developed a procedure in which treatment with arginase is followed by the use of xanthydrol for the estimation of the resulting urea. Thus far these methods have not been adapted to the determination of free arginine in tissue extracts. It is indeed unlikely that the Jansen and Hunter-Dauphinee methods could conveniently be applied to biological material. The preformed ammonia content would have to be known with precision and in some tissues, such as muscle, considerable uncertainty attends its determination due to the lability of its precursors. Hunter and Dauphinee have also pointed out that the liver extract used as arginase exercises a deaminizing action upon protein hydrolysates. This is to be recognized as a potential source of error in application of the method under conditions different than those specified by Hunter and Dauphinee. Furthermore, the arginine content of the materials that we have examined is far too low to permit analysis by these methods. By resorting to the volumetric method of Allen and Luck (15) for estimation of dixanthydryl urea, it has been possible to develop a procedure, in principle resembling that of Bonot and Cahn and applicable to the determination of very small amounts of arginine. Urea is determined simultaneously.

Reagents

Sodium tungstate, 10 per cent solution (16).

Sulfuric acid, 0.66 N.

Carbonate-bicarbonate buffer, 1 M, pH 9.5. To 500 cc. of 1.0 M sodium bicarbonate add a molar solution of sodium carbonate. Continue adding the latter until pH 9.5 is attained.

Arginase. (a) *Stock solution*, a glycerol extract of rabbit liver, prepared according to the method of Hunter and Dauphinee (17). This will retain its activity for several months. (b) *Dilute solution.* To 1 cc. of the stock solution add 9 cc. of water. Prepare daily.

Copper sulfate solution, 20 per cent. Dissolve 20 gm. of copper sulfate ($CuSO_4 \cdot 5H_2O$) in water and dilute to 100 cc.

Saturated solution of barium hydroxide, approximately 7 per cent $Ba(OH)_2 \cdot 8H_2O$.

Glacial acetic acid.

Solution of xanthydrol in methyl alcohol. Suspend 10 gm. of xanthydrol in 90 gm. of absolute methyl alcohol (113 cc.). Shake vigorously. After 2

days filter through dry paper into a narrow mouthed, well stoppered, amber bottle.

Saturated solution of dixanthydryl urea in methyl alcohol. Suspend 10 to 20 mg. of dixanthydryl urea (15) in 1 liter of absolute methyl alcohol contained in a well stoppered bottle. Shake at intervals during several days. Filter in portions as needed.

Standardized potassium dichromate, 1.0 N.

Concentrated sulfuric acid, 36 N.

Standardized sodium thiosulfate, 0.1 N.

Potassium iodide, 10 per cent solution.

Starch solution as indicator.

Procedure

Thus far in our experimental work we have confined the method, now to be described, to the determination of arginine in muscle, liver, and whole carcass.

Weigh 6 gm. of the frozen powdered tissue (18) into a large weighing bottle (40 × 80 mm.). Add 42 cc. of ice water, 6 cc. of ice-cold 10 per cent sodium tungstate, and 6 cc. of ice-cold 0.66 N sulfuric acid. Shake vigorously between additions. Permit the contents to stand with occasional shaking for about 3 minutes. Filter. Transfer 15 cc. of the filtrate to a 25 cc. volumetric flask. Add 2 cc. of the carbonate-bicarbonate buffer and 3 cc. of the diluted arginase solution. Transfer a second portion of 15 cc. to another 25 cc. volumetric flask and add 2 cc. of the carbonate-bicarbonate buffer but omit the arginase. Of the two samples thus prepared, the latter is to serve for the estimation of the preformed urea. It may be designated "control." The former, which contains arginase, serves in determination of the total urea (preformed urea plus urea from arginine). We usually refer to it as the "experimental."

Transfer both flasks to a thermostat at 25°. After standing 18 to 21 hours add to each 1 cc. of 20 per cent copper sulfate and 3 cc. of saturated barium hydroxide. Add the latter in portions of 8 or 10 drops and shake well between additions. Dilute each to 25 cc., shake, and filter through dry paper.

Place 5 cc. of the filtrate in a 15 cc. centrifuge tube and add 5 cc. of glacial acetic acid and 0.5 cc. of the methyl alcohol solution of xanthydrol. Mix the contents intimately, either by the use of a stirring rod or by closing the tube with a paraffined cork and shaking vigorously. Allow to stand for 1 hour during which time the

dixanthydryl urea separates slowly from solution.   If much be present it gathers in large loose clumps.   Centrifuge for 10 minutes at about 2500 R. P. M.   Decant off the supernatant fluid and wash the residue with a saturated solution of dixanthydryl urea in methyl alcohol.   Unless the urea and arginine content of the tissue be unusually high a single washing with 15 cc. of the methyl alcohol is sufficient.   If the amount of dixanthydryl urea exceeds 2 mg. (a quantity easily recognized with practise), a second washing is advisable.   It is important that the precipitate be broken up thoroughly to insure complete washing.   Again centrifuge, this time for 5 minutes, and pour off the supernatant fluid.   The excess of alcohol which clings to the precipitate and the wall of the tube is removed by drying.   This may be accomplished by placing in an oven for 1 hour at 100° or by permitting the samples to remain overnight in a warm dry room.

To the dry tube containing the derivative add 3.00 cc. (accurately measured) of the standardized potassium dichromate followed by 5 cc. of concentrated sulfuric acid.   Mix intimately by means of a rod.   After 2 to 3 minutes wash the contents quantitatively into a 250 cc. Erlenmeyer flask with 60 to 75 cc. of distilled water.   Add 10 cc. of 10 per cent potassium iodide and titrate with 0.1 N sodium thiosulfate in the usual fashion with a few drops of starch solution as indicator.

Calculation of Results

If X_c cc. of thiosulfate be used in the titration of the control then $(30 - X_c)$ cc. represents the thiosulfate equivalent of the dichromate consumed in oxidation of the dixanthydryl urea. (This assumes that the potassium dichromate and sodium thiosulfate are exactly 1.0 N and 0.1 N respectively.)   Since 1 cc. of 0.1 N thiosulfate is equivalent to 0.0518 mg. of urea, $(30 - X_c)$ cc. corresponds to $(30 - X_c) \times 0.0518$ mg. of urea.   Likewise if X_e cc. of thiosulfate be used in titration of the experimental, $(30 - X_e) \times 0.0518$ mg. is the corresponding urea value.   From the former it follows that the preformed urea content of the tissue is $\frac{100}{6} \times \frac{60}{15} \times \frac{25}{5} \times (30 - X_c) \times 0.0518$ mg. per 100 gm.   In similar fashion it may be shown that the total urea content (arginine urea plus preformed urea) is $\frac{100}{6} \times \frac{60}{15} \times \frac{25}{5} \times (30 - X_e) \times 0.0518$ mg. per 100 gm. of tissue.   The urea derived from arginine

is therefore equal to $\frac{100}{6} \times \frac{60}{15} \times \frac{25}{5} \times (X_c - X_e) \times 0.0518$ mg. per 100 gm. of tissue. Since 1 mg. of urea is formed from 2.9 mg. of arginine, the arginine content of the tissue is $\frac{100}{6} \times \frac{60}{15} \times \frac{25}{5} \times (X_c - X_e) \times 0.0518 \times 2.9$ mg. per 100 gm. of tissue.

Analysis of Aqueous Solutions of Arginine

In the first experiments the estimation of arginine in aqueous solutions of known strength was undertaken. The technique employed was different in several respects than that ultimately developed for tissue analysis but the results were considered of value in demonstrating that the salient characteristics of the method were sound.

50 mg. of d-arginine carbonate, prepared from gelatin by the flavianic acid method of Pratt (19), were dissolved in 50 cc. of phosphate solution, pH 7.5, 0.04 M. 10 cc. of dilute arginase were added and the solution allowed to stand overnight at room temperature. 25 cc. portions were then treated with 1 cc. of 10 per cent sodium tungstate, 1 cc. of 0.66 N sulfuric acid, and 2 cc. of dialyzed iron (Merck's 5 per cent). The filtrates were analyzed for urea by dilution of 1 cc. and 2 cc. samples to 5 cc., addition of glacial acetic acid and xanthydrol, and estimation of the resulting dixanthydryl urea as described. In later experiments the phosphate buffer of pH 7.5 was replaced by a glycine buffer, 0.1 M, of pH 9.5, in accordance with the findings of Edlbacher and associates (9, 20) and Hunter and Morrell (21). The carbonate-bicarbonate buffer was used in Experiment 5. Analyses were generally made in quadruplicate. The results are presented in Table I.

In two other experiments attempts were made to analyze the digestion mixture directly, by omission of the treatment with sodium tungstate, sulfuric acid, and colloidal iron. Low results were obtained. Likewise omission of the clarification with colloidal iron was found to be impermissible.

Arginase Preparation

Before proceeding with experiments on tissue extracts, it was thought advisable to compare several different preparations of arginase. It occurred to us that acetone precipitation of the

glycerol extract might yield a dry product of greater purity and stability.

20 cc. of the stock solution of arginase were poured into 200 cc. of ice-cold acetone. The precipitate was fractionated into a

TABLE I

Estimation of Arginine in Aqueous Solution

Experiment No.	Buffer	Recovery of arginine	
		1 cc. samples	2 cc. samples
		per cent	*per cent*
1	Phosphate, pH 7.5	100.3	100.9
		99.6	106.9
		93.5	106.7
		99.6	104.2
2	Glycine, pH 9.5	95.1	95.8
		94.6	97.0
		93.3	
		94.3	
3	Glycine, pH 9.5	103.8	106.4
		103.1	106.7
		103.5	107.2
		100.7	107.0
4	Glycine, pH 9.5	97.1	97.2
		97.1	99.2
		100.3	100.1
		97.6	100.2
5*	Carbonate, pH 9.5	105.3	98.5
		106.0	99.4
		100.9	106.7
		100.8	107.8

* In this experiment 39.5 mg. of arginine carbonate and 11.3 mg. of urea were employed instead of 50 mg. of arginine carbonate.

water-soluble and a water-insoluble fraction. The activities of these preparations were then compared with that of the initial glycerol extract. Quantities of arginine (varying from 0.5 to 2.0 mg.) were employed in the comparison. The results indicated clearly that acetone treatment of the glycerol extract is inadvisable.

Buffer

As soon as attempts were made to determine arginine in tissue extracts several changes in procedure became necessary. Colloidal iron was replaced by copper sulfate and barium hydroxide for the clarification of the tungstic acid extracts. Filtration after the use of colloidal iron was found to proceed very slowly and much of the fluid desired for analysis was retained by the bulky gelatinous precipitate. Treatment with copper sulfate and barium hydroxide yielded a filtrate of greater volume in which urea could be determined without difficulty by the xanthydrol method.

The nature of the buffer which was added with arginase to the tungstic acid extracts proved to be of greater importance than first anticipated. 0.1 M glycine (2 cc. to 15 cc. of the tungstic acid extract) yielded values which tended to be inconstant and low. We suspected that the concentration of the glycine was insufficient to maintain the pH within the desired limits. The use of more concentrated glycine was attended by other difficulties which eventually led us to compare 0.1 M glycine, 1.0 M borate, and 1.0 M carbonate,—all of pH 9.5.

15 cc. portions of the tungstic acid extract of rat muscle were transferred to 25 cc. volumetric flasks. To each were added 3 cc. of dilute arginase. The first was buffered with 2 cc. of 0.1 M glycine (Sorensen), the second with 2 cc. of 1.0 M borate, and the third with 2 cc. of 1.0 M carbonate-bicarbonate. All three were permitted to stand for 18 hours at 25° and then clarified with 1 cc. each of copper sulfate and saturated barium hydroxide. The filtrates were analyzed for urea. Controls, lacking arginase, were set up for determination of the preformed urea. Values of 10.0, 17.5, and 24.3 mg. per 100 gm. of muscle were found for the arginine content of samples buffered with glycine, borate, and carbonate respectively. A second experiment yielded corresponding average values of 14.2, 26.6, and 37.9. It is to be observed that the largest arginine values were obtained in the samples buffered with carbonate. We concluded that incomplete hydrolysis of the arginine proceeded in the samples buffered with glycine and borate; a conclusion which was supported by observations on the recovery of added arginine.

Extraction with Tungstic Acid

If the estimation of the preformed urea in tissues is not desired the elaboration of a procedure for the direct determination of arginine would render superfluous the control determinations described in the preceding paragraphs and would expedite analysis. We attempted, therefore, to devise a method which would not only accomplish the desired end but would serve as a check upon the indirect procedure previously outlined.

The principle employed was that of heat coagulation for removal of the tissue proteins and the use of urease for destruction of the performed urea. 6 gm. of frozen, powdered muscle were weighed out and transferred to a boiling tube (8″ × 1″) with approximately 40 cc. of boiling 0.01 N acetic acid. The tube was immersed in a bath of boiling water for 7 minutes and then cooled. Normal sodium hydroxide was added until the mixture was very faintly alkaline to phenolphthalein (*circa* pH 8). 5 cc. of 0.5 per cent urease (Van Slyke and Cullen (22)) dissolved in 0.1 M phosphate buffer (pH 7.0) and filtered through glass wool were added. After standing for 30 minutes at 45° the tube was placed in boiling water for 5 minutes for inactivation of the urease. The contents were cooled, diluted to 50 cc., and filtered. To 20 cc. of the filtrate were added 5 cc. of 1.0 M buffer (pH 9.5) and 5 cc. of diluted arginase. The sample was maintained overnight at 25° and then clarified with copper sulfate and baryta. The mixture was diluted to 50 cc. and filtered. The urea, all of which was presumably derived from arginine, was estimated as dixanthydryl urea in the usual fashion. A second 6 gm. sample of the same tissue powder was analyzed simultaneously by the method of tungstic acid extraction and indirect estimation of the arginine as described under "Procedure." A glycine buffer was used in one experiment and the carbonate-bicarbonate buffer in the remainder. In several experiments known quantities of arginine were added and the per cent recovery determined. The arginine was added in aqueous solution to the weighed tissue samples, the water thus added being subtracted from the 42 cc. employed in the tungstic acid procedure or from the 0.01 N acetic acid used in the heat coagulation method. Analyses were made either in duplicate or triplicate. The results are presented in Table II. It is apparent that the arginine values as determined by the heat coagulation

method are too low; a conclusion which is supported by the unsatisfactory recovery of added arginine. In the tungstic acid method, on the contrary, the extra arginine was fairly well accounted for.

Arginine Content of Liver, Muscle, and Carcass in Fasting Rats

The distribution of arginine in the normal animal was determined by analysis of the muscle, liver, and liver-free carcass of the fasting rat.

TABLE II

Comparison of Tungstic Acid and Heat Coagulation Methods. Recovery of Added Arginine

Experiment No.	Arginine added per 100 gm. muscle*	Preformed arginine per 100 gm. muscle*		Total arginine per 100 gm. muscle*			Difference between (A) and (B)
		Tungstic acid method	Heat coagulation method	Calculated (A)	Found (B)		
					Tungstic acid method	Heat coagulation method	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
1†	0	34.6	20.8				
2	0	47.9	19.7				
3	184.7		14.4	199.1		154.4	−23.0
4	104.0	56.8		160.8	156.7		− 2.6
5	175.4	53.5		228.9	231.0		+ 0.9
6	175.4	17.4		192.8	194.6		+ 0.9
7*	177.5	55.9		233.4	217.2		− 6.9
8*	160.7	18.6		179.3	195.3		+ 8.9

* Whole carcass was used instead of muscle in Experiment 7. Carcass from which the liver had been removed was used in Experiment 8.

† Glycine was used as buffer in place of carbonate-bicarbonate.

Twelve females, each of about 190 gm., were used. The animals were drawn from several closely related litters and were maintained after weaning upon a stock ration of cracked wheat, ground oats, yellow corn-meal, flaxseed meal, alfalfa meal, whole milk powder, yeast, bone meal, sodium chloride, and cod liver oil (23). They were fasted, without access to feces, for 36 hours before analysis.

The animals were stunned and bled through a deep thoracic incision. The liver was rapidly excised and immersed in liquid air. From six of the animals the thigh and buttock muscles of

one limb were then removed and frozen. From the remaining six animals muscle samples were not removed. Instead the liver-free carcass, including the blood which drained from the thorax, was finely minced by freezing and grinding. About 20 gm. of the product were transferred to a mortar containing liquid air

TABLE III

Distribution of Arginine in Fasting Rats

Litter No.	Weight of rat	Arginine content per 100 gm. liver-free carcass		Litter No.	Weight of rat	Arginine content per 100 gm. muscle	
	gm.	*mg.*	*mg.*		*gm.*	*mg.*	*mg.*
9	190	32.3		19	190	28.3	
		17.2	27.9			29.0	28.7
		34.3					
21	170	24.2		19	250	16.6	
		26.2	26.2			18.6	17.6
		28.2					
21	190	27.2		19	185	29.8	
		26.6	27.3			30.3	30.1
		28.2				30.3	
19	210	19.9		19	205	12.3	
		17.4	18.6			5.8	7.7
		18.4				4.8	
19	180	34.8		19	200	29.5	
		29.1	37.6			28.5	29.2
		49.0				29.5	
				19	195	40.3	
						40.7	43.1
						48.4	
Average...............			27.5				26.1

and finely powdered. Of the frozen muscle, liver, and carcass powders thus obtained, 6 gm. samples were weighed out and analyzed in triplicate for arginine. The results of the analyses are presented in Table III. The values for liver have been deliberately omitted because of fluctuations, probably due to the great activity of the arginase in the macerated organ, which gave

to the liver values an uncertain significance. The determinations on muscle and the liver-free carcass were free of the difficulties encountered in analysis of the liver.

It should be pointed out that an arginine content of 25 or 30 mg. per 100 gm. of tissue corresponds to 2 or 2.5 mg. of arginine α-amino nitrogen. This in turn represents 4 or 5 per cent of the non-protein amino nitrogen content of rat muscle, liver, or carcass. If it be assumed that all of the non-protein amino nitrogen is present in the form of amino acids with a mean molecular weight of 120, the content of the latter would be about 390 mg. per 100 gm. of tissue. From this it follows that arginine would account for about 6 or 7 per cent of the non-protein amino acids of rat muscle and carcass.

It is also of interest that these analyses demonstrate clearly the existence of arginine as a constituent of vertebrate muscle. This is not in harmony with the conclusions of Kutscher and Ackermann (24), who, in summarizing their extensive investigations on the extractives of muscle expressed the generalization that arginine, though a characteristic constituent of invertebrate muscle, was absent from the vertebrates. We consider it likely that any method of direct isolation, such as that employed by Kutscher and Ackermann, would fail to detect the small quantities of arginine reported by us in this paper. Furthermore, it is conceivable that the species investigated by Kutscher and Ackermann may have contained quantities of arginine even smaller than that in rat muscle. We have found, for example, that two rattlesnakes (*Crotalus oregonus*) which were killed and analyzed on the 4th day after capture contained surprisingly little arginine. Although the non-protein amino nitrogen content was twice that of rat muscle, the arginine content was only 9.9 mg. in one specimen and less than 2 mg. per 100 gm. of muscle in the other.

PART II

Catabolism of Arginine

Most of the studies on the metabolism of arginine, as recorded in the literature of biochemistry, have been concerned with the rôle of arginine as a precursor of creatine and the purines. A little attention has been given to its significance as a source of glucose in the diabetic animal. Other inquiries have been devoted to its

nutritive importance. We recall, however, only one series of investigations upon the magnitude and rate of urea formation in the animal body following the injection of this amino acid (25). In connection with our studies of amino acid metabolism we were especially desirous of extending these early observations by methods that would permit comparison of the data with results gained from other amino acids.

In the experiments now to be described we have studied the decrease in amino acid nitrogen and the rate of formation of urea after injection of standard fasted rats with arginine.

Procedure

Standard white rats, drawn from our own colony, were used as the subjects of experiment. They were maintained, prior to use, upon the stock diet described in Part I.

Two groups of females, the first consisting of eight animals and the second of ten, were employed. The animals weighed between 115 and 150 gm. on the day of experiment. 1 week before use the members of a group were selected and caged together. 36 hours before the experiment food was withdrawn and a coarse screen bottom inserted in the cage to prevent access to the feces. On completion of the fast each rat was weighed, and its bladder emptied by treatment with ether (26). Of the eight or ten animals in the group, all but two or three were then injected with the arginine solution. The remainder which was designated controls received 0.9 per cent sodium chloride.

Each animal was injected immediately after the emptying of the bladder. The time was recorded and the animal transferred to an individual metabolism cage. The times of injection were so arranged as to leave intervals of at least 30 minutes between killings. The members of the group were then killed in prearranged order so that intervals of 0, 1, 2, 3, 5, 8, and 12 hours occurred between the times of injection and the times of killing. The entire carcass, inclusive of all postinjection excreta, was analyzed for amino acid nitrogen and urea. The controls were analyzed after 1, 8, and 12 hours.

d-Arginine carbonate, prepared by the method of Pratt, was used. For each group of animals, 7.6 gm. of the arginine carbonate were dissolved in water, the solution neutralized to litmus

with hydrochloric acid, and made up to 32 cc.   2.86 cc. per 100 gm. of body weight were injected in each animal.   This was equal to approximately 0.4 gm. of amino acid nitrogen per kilo.

All injections were subcutaneous, generally in the mid-lumbar region.

For individual metabolism cages we used a simple arrangement consisting of a large funnel inverted over a No. 8 evaporating basin. The latter was covered with a coarse screen upon which the animal was placed.   The arrangement was particularly convenient in that the evaporating basin into which the excreta passed served also to receive the minced carcass as mentioned later in the procedure.   A trace of mercuric chloride was added to prevent hydrolysis of urinary urea by fecal urease which, conceivably, might be present.

The animals were stunned and passed immediately through a heavy mincer.   The mincings were received in liquid air contained in a No. 8 evaporating basin[1] which was used in alternation with the basin containing the postinjection excreta.   After three or four mincings with a coarse blade the product was passed two or three times through a fine blade.[2]   Finally 15 or 20 gm. of the product were transferred to an iron mortar with liquid air and there reduced by pounding to a very fine powder.   Of the latter a 7 gm. sample was weighed into a bottle (40 × 80 mm.) and treated with 49 cc. of ice water, 7 cc. of ice-cold 10 per cent sodium tungstate, and 7 cc. of ice-cold 0.66 N sulfuric acid.   The filtrate was analyzed for amino acid nitrogen by the method of Kiech and Luck (18), and for urea by the xanthydrol method of Allen and Luck (15).   When the urea values were very high, as in later members of the series, it was sufficient to use 1 or 2 cc. portions of the clarified filtrate and to dilute these with water to 5 cc. before adding the acetic acid and xanthydrol.   •

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[1] We have found recently that freezing of the mincings to the cold basin can readily be prevented by painting the dish lightly with melted paraffin.

[2] Unless the mincer is thoroughly chilled the fine blades are inclined to become packed and jammed, especially if the mincings are solidly frozen. Difficulty is avoided if the liquid air is used sparingly in the later stages, in such a way that the tissue is kept in the vicinity of the freezing point without being fully and solidly frozen.

Results

The findings have been presented graphically in Fig. 1. It should be pointed out that the control values agree well with averages which we have computed from seven related experiments of similar nature involving twenty-one control animals.[3]
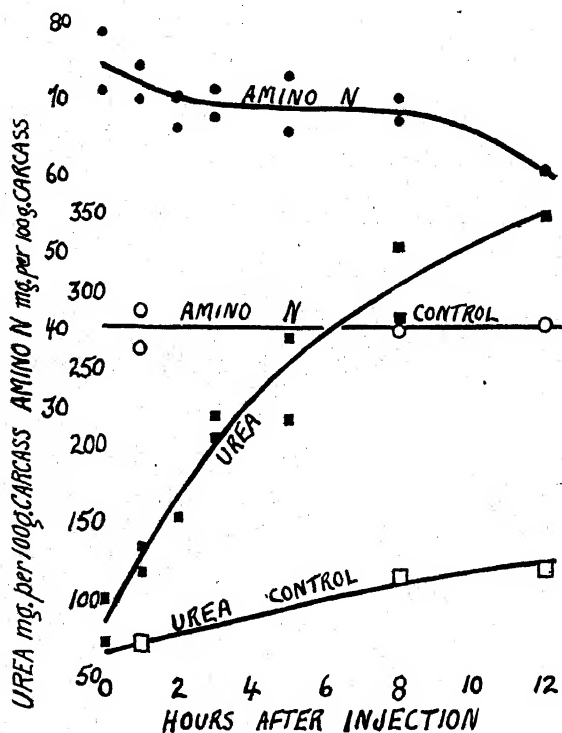
FIG. 1. Rate of urea formation and amino nitrogen decrease in rats injected with arginine.

In considering the possibilities involved in the metabolism of arginine it is apparent that a small decrease in the amino nitrogen content of the animals from the high postinjection level may indicate either of two paths of metabolism. It may mean that little of the injected arginine has been deaminized, most of the substance either being retained as arginine or being excreted as

[3] These composite averages are for amino acid nitrogen: 1 hr., 43.3; 7 hrs., 42.2; 12 hrs., 42.2; for urea: 1 hr., 70.7; 7 hrs., 101.5; 12 hrs., 116.6.

such. On the contrary it may indicate that ornithine, however rapidly it may form, undergoes partial deamination with equally great rapidity. Otherwise the amino nitrogen content of the animals would actually increase for a time, since the arginine molecule possessed of one reactive group would be replaced by ornithine possessed of two.

Actually the slow decrease in the amino acid content of the injected animals coupled with the rapid and extensive formation of urea can only mean, in our judgment, that the latter possibility is more nearly in accordance with the facts.

It is possible, indeed, by assuming arginine to be the only guanidine compound that can be hydrolyzed in the animal organism with the production of urea, to calculate from the data of Fig. 1 the approximate quantity of arginine remaining unmetabolized at any given time. An estimate may also be formed of the amount of undetermined nitrogen (inclusive of ornithine). The calculations reveal that within 12 hours 72 per cent of the injected arginine had disappeared. At no time, however, did the undetermined nitrogen (including that of ornithine) amount to more than 10 per cent of the injected nitrogen. The inference, therefore, is that however rapidly arginine may be catabolized, ornithine, which presumably arises as an intermediate, disappears with almost equal rapidity. In the experiments reported in Part III it has been possible by the direct determination of arginine to demonstrate the validity of this conclusion.

PART III

Catabolism of Arginine as Studied by the Determination of the Residual Arginine

Procedure

Sixteen female rats weighing from 174 to 216 gm. on the day of experiment were used. The preexperimental treatment of the animals was in all essentials the same as that given the animals used in the preceding investigation, the principal difference being that the animals were studied in eight groups of two each in place of two large groups.

On the day of experiment the animals were injected with a neutralized aqueous solution of arginine carbonate. The quantity

employed was much less than in the preceding experiment.   There it will be recalled the amount injected was approximately 0.68 gm. of arginine carbonate per 100 gm. of body weight.   In this investigation each animal received only 0.30 gm. of arginine carbonate, equivalent to about 0.15 gm. per 100 gm. of body weight.   This reduction was rendered possible by the low concentration of arginine existing preformed in the animal body and by the sensitivity of the analytical method used in its determination. Had this smaller quantity of arginine been used in the experiments of Part II, the amino nitrogen content of the animals would have been elevated at the time of injection from a basal level of 40 mg. per 100 gm. of body weight up to only 50 mg.   The latter value does not lie much above the upper limit for the amino nitrogen content of the normal animal, and in consequence attempts to study amino nitrogen changes in the injected animals would have been futile.

The specified quantity of arginine carbonate, dissolved in 5 cc. of 0.9 per cent sodium chloride was injected subcutaneously. The controls received the sodium chloride solution without arginine.

Mercuric chloride was, of necessity, not added to the collecting basins constituting part of the individual metabolism cages.

Owing to the activity of liver arginase which, we feared, might hydrolyze arginine during the preparation of the sample, the separate analysis of the liver was considered advisable.   Accordingly, after killing an animal, the liver was rapidly excised and immersed in liquid air, while the liver-free carcass with the blood and postinjection excreta was minced, frozen, and sampled.

Arginine was determined in the tungstic acid extracts of liver and carcass by the method described in Part I and urea by that of Allen and Luck (15).   In calculating the arginine content of the entire animal the total arginine content of the whole liver was added to that of the liver-free carcass.   The sum, multiplied by 100, and divided by the weight of the animal gave the arginine content in mg. per 100 gm. of body weight.

Results

In Fig. 2 we have presented the results of the experiment.   Each point on the curves represents the average of triplicate analyses

upon a single animal.   Two urea samples had to be discarded because of loss of urine when the animal was removed from the metabolism cage for killing.

It has seemed unnecessary to plot the arginine and urea values obtained on analysis of the liver.   It is sufficient to mention that all the arginine values fell between 7.6 and 35.4.   Presumably any tendency towards the storage of arginine in the liver was
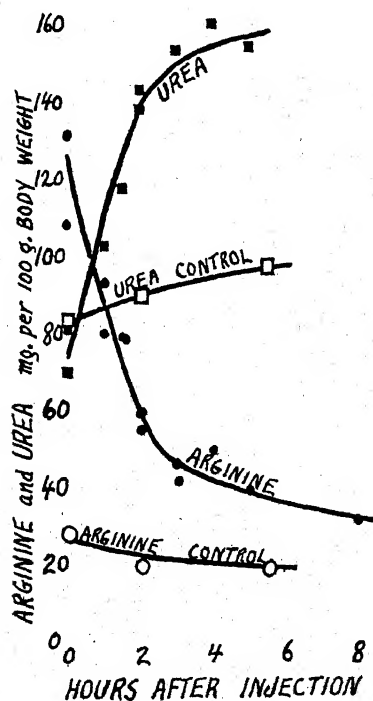
FIG. 2. Rate of urea formation from arginine in injected rats

overcome by the activity of liver arginase.   The values for the urea content of the liver, starting from 28, rose to 63 within 1½ hours and remained at about that level for the duration of the experiment.

By examination of Fig. 2 it is possible to calculate the relative amounts of arginine, urea, and undetermined nitrogen present at any given time.

Total N injected as arginine, *mg. per 100 gm. animal*......... 36
Extra " present "   "   after *t* hours, *mg. per 100 gm. animal*.   *b*
    "   "   "   " urea after *t* hours, *mg. per 100 gm. animal*...   *a*
    "   "   "   in undetermined form after *t* hours, *mg. per 100*
    gm. animal... *x*

Since 36 mg. of nitrogen were injected, $x = 36 - (a + b)$.

The calculated values for x, at 1, 3, and 5 hours after injection of arginine, are represented in Table IV. The values for b were obtained from Fig. 2 by multiplying the differences between

TABLE IV

Amounts of Arginine and Its Metabolism Products in Rats at Various Times after Subcutaneous Injection with Arginine

Time	Mg. per 100 gm. animal			Per cent of total nitrogen injected		
	a	*b*	*x*	Urea N	Arginine N	Undetermined N
hrs.						
1	11.4	20.3	4.3	31.7	56.4	11.9
3	26.9	8.0	1.1	74.7	22.2	3.1
5	28.3	6.1	1.6	78.7	16.9	4.4

corresponding points on the two curves designated arginine, and arginine control, by $\frac{5.6}{17.4}$. The a values were obtained in like fashion from the urea curves by multiplication of the corresponding differences by $\frac{2.8}{8.5}$.

Reference to Table IV makes it clear that little nitrogen is present in the form of ornithine, or any degradation product of ornithine. After the first hour the quantity is insignificant. The observations and deductions therefore confirm the conclusions of Part II. In only one important respect are these results different; the arginine content of the animals approaches the fasting level much more rapidly than in the preceding experiments. This is probably due to the injection of much less arginine.

Although it is to be expected that arginine, added to macerated liver, would disappear with great rapidity it does not follow that arginine, *in vivo*, would be metabolized equally as fast. Indeed, as we shall show in a later paper, the rate of catabolism of injected

arginine is not appreciably greater than that of certain other amino acids. Our findings suggest that ornithine might be expected to undergo catabolism still more rapidly.

We propose to correlate these and related observations on the metabolism of amino acids with data on oxygen consumption and carbon dioxide production.

SUMMARY

1. A volumetric method is described for the determination of arginine in protein-free tissue extracts. The arginine is converted in the presence of arginase to ornithine and urea and the latter determined as dixanthydryl urea by oxidation with potassium dichromate and sulfuric acid.

2. In fasting female rats the arginine content was found to average 26.1 mg. per 100 gm. of muscle, and 27.5 mg. per 100 gm. of carcass.

3. Arginine, administered to fasting rats by subcutaneous injection, was rapidly catabolized. Less than 12 per cent of the injected nitrogen was present at any time in substances other than urea and arginine. Ornithine, which presumably would be formed, must have been catabolized almost as rapidly as arginine.

The data presented in Parts I and III were drawn from the thesis presented by Anna Evelyn Smith in partial fulfilment of the requirements for the degree of Master of Arts. The experimental work in Part II was performed by Veon Carter Kiech.

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BLOOD AS A PHYSICOCHEMICAL SYSTEM

X. THE PHYSICOCHEMICAL PROPERTIES OF OXYGENATED HUMAN BLOOD

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The present communication comprises one part of a generalized quantitative description of the physicochemical properties of human blood. Except in the first section, it is concerned exclusively with oxygenated blood of standard chloride content and standard concentration of hemoglobin in cells. The independent variables fully accounted for are base, so far as involved in variations of the acid-base equilibrium, oxygen capacity, and pressure of carbon dioxide. In part, variation in serum protein content is also accounted for.

I

This section consists of an estimate of the buffer value of plasma, deduced from the study of ten specimens taken from six individuals.

For each specimen five points on the carbon dioxide dissociation curves, at the temperature 37.5°, were experimentally determined (with two exceptions, in duplicate or triplicate) the total number of separate determinations being 107. From the data, values of $C_{H_2CO_3}$ and C_{BHCO_3} (concentrations of free and combined carbonic acid in milli-equivalents per liter) and of pH were calculated, with the relations

$$C_{H_2CO_3} = 0.031 \times pCO_2$$

$$C_{BHCO_3} = C_{total\ CO_2} - C_{H_2CO_3}$$

$$pH_s = 6.10 + \log C_{BHCO_3} - \log C_{H_2CO_3}$$

The protein concentrations C_P (gm. per liter) were also estimated and values of the quantity $\dfrac{C_{BHCO_3}}{C_P}$ were computed for all cases. These values were then plotted against the values of pH separately for each specimen of plasma, and curves were fitted in each case
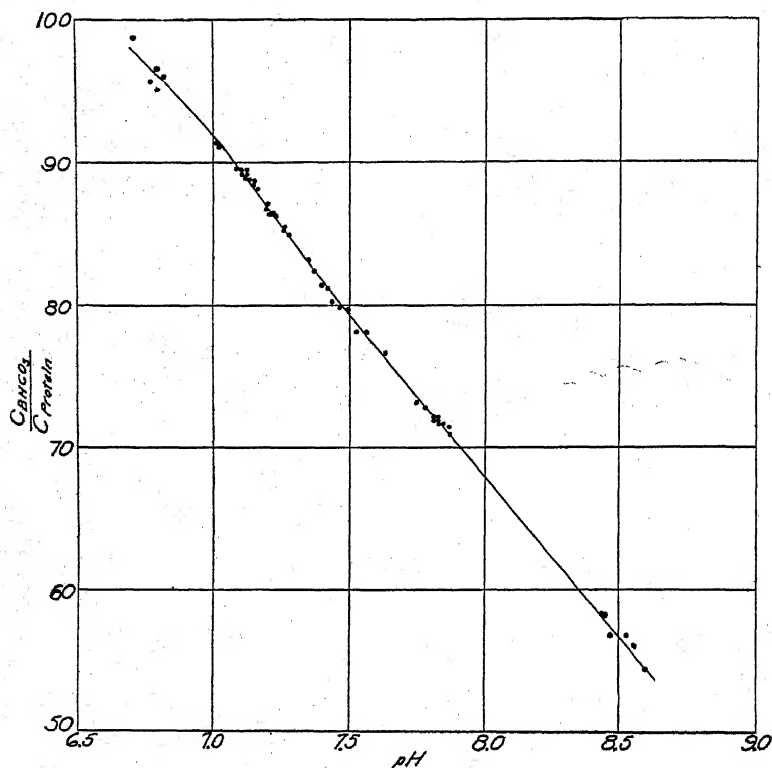
Fig. 1. Relation of $\dfrac{C_{BHCO_3}}{C_{protein}}$ to pH in ten specimens of human plasma

to the points.   Next the value of $\dfrac{C_{BHCO_3}}{C_P}$ for pH = 7.4 in each of the ten specimens of plasma was read from the curve, the difference between this value and an arbitrarily chosen standard value was computed, and this difference was subtracted from each of the five $\dfrac{C_{BHCO_3}}{C_P}$ values of the specimen.   The resulting 50 values

were then plotted against pH on one chart. This is shown as Fig. 1.

Aside from variations in concentrations of base and of proteins the acid-base properties of these ten specimens of human blood plasma seem to be nearly identical. They are summed up in Table I, Columns 5 and 6, in which the fiction is adopted of assigning all buffer action to protein and carbonic acid, *i.e.*

$$C_{BHCO_3} + C_{BP} = B = \text{constant}$$

It should be noted that from Column 6 the unknown value of $\dfrac{C_{BP}}{C_P}$ for pH = 6.8 has been subtracted.

Van Slyke and his associates (1) have found for the normal mixture of serum proteins of the horse the constant buffer index of 0.104. Our results for human blood plasma are in excellent agreement with this measurement, since our mean value for the range of pH between 6.8 and 8.0 is 0.105, for the range between 7.0 and 7.8, 0.109, and a result a little larger than 0.104 is to be expected on account of the presence of small quantities of other substances which exert buffer action.

There seems to be no doubt that the relation between $\dfrac{C_{BP}}{C_P}$ and pH is not precisely linear and that the buffer index of human blood plasma passes through a maximum in the physiological range, or more precisely, at some point between the values 7.10 and 7.45 for pH.

II

This section consists of an estimate of the buffer value of oxygenated human erythrocytes. The value has been deduced from the study of seven specimens of blood taken from five individuals.

Each specimen was equilibrated at the temperature 37.5° with atmospheres containing oxygen in sufficient amount to insure complete oxygenation at two or more different pressures of carbon dioxide. The total number of equilibrations was seventeen. In each instance the values of the following quantities were estimated in duplicate or triplicate: (1) pressure of carbon dioxide, (2) total carbonic acid of blood, (3) total carbonic acid of plasma, (4) cell volume. Hemoglobin concentrations per liter of blood and serum protein concentrations per liter of blood were also determined.

The computations were carried out as follows, on the assumption that the approximation for whole blood $C_{BHCO_3} + C_{BHbO_2} + C_{BP_r} = B = $ constant. First, taking $pK' = 6.10$, values of pH_s
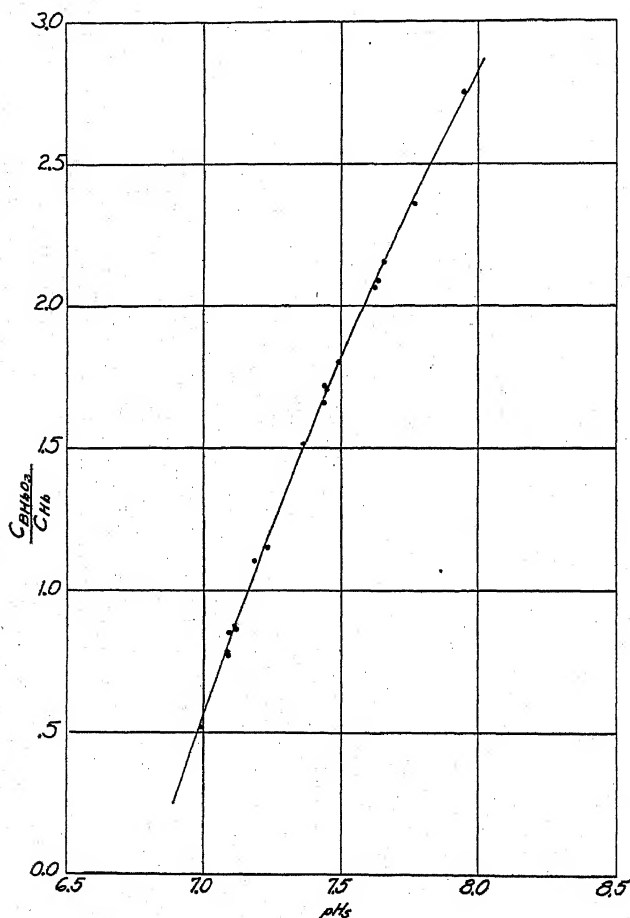
FIG. 2. $\dfrac{C_{BHbO_2}}{C_{Hb}}$ as a function of pH_s in oxygenated human blood

were obtained. Secondly, from these pH_s values, the estimates of serum protein concentrations, and the values of $\dfrac{C_{BP}}{C_P}$ (Table I, Column 5), values of C_{BP_r} were obtained. Thirdly, values of

C_{BHCO_3} were computed from the data.   Next, values of C_{BHCO_3} + $C_{\text{BP}_s} = \text{B} - C_{\text{BHbO}_2}$ were tabulated and, at length, values of $\dfrac{\text{B} - C_{\text{BHbO}_2}}{C_{\text{Hb}}}$.

The values of this last quantity for each specimen of blood were plotted against values of pH_s and these graphs were then brought to the same arbitrarily chosen level by means similar to those employed in the preceding section for the quantity $\dfrac{C_{\text{BHCO}_3}}{C_{\text{P}}}$. This result is shown in Fig. 2.

The equation of the curve of Fig. 2 is

$$\frac{C_{\text{BHbO}_2}}{C_{\text{Hb}}} = \text{N} + 9.75 \text{ pH}_s - 0.5 \text{ pH}_s^{+2}$$

If, for convenience, we compute the value of $\dfrac{C_{\text{BHbO}_2}}{C_{\text{Hb}}}$ as that quantity by which, for $\text{pH}_s = x$, the value exceeds the value for $\text{pH}_s = 6.8$, we have

$$\frac{C_{\text{BHbO}_2}}{C_{\text{Hb}}} = 9.75 \text{ pH}_s - 0.5 \text{ pH}_s^{+2} - 43.18$$

The buffer index, *referred to pH_s*, of oxygenated red blood cells per mM of oxygen capacity is accordingly

$$\frac{1}{C_{\text{Hb}}} \cdot \frac{dC_{\text{BHbO}_2}}{d\text{pH}_s} = 9.75 - \text{pH}_s$$

For these seven specimens of oxygenated blood the mean value of the relation between pH_s and pH_c is defined by the equation $(\text{pK}_c' = 5.97)$ $\text{pH}_s = 1.2658\text{pH}_c - 1.6202$ (see Table I, Columns 1 and 2), whence

$$\frac{d\text{pH}_s}{d\text{pH}_c} = 1.2658$$

Accordingly

$$\frac{1}{C_{\text{Hb}}} \cdot \frac{dC_{\text{BHbO}_2}}{d\text{pH}_c} = \frac{1}{C_{\text{Hb}}} \cdot \frac{dC_{\text{BHbO}_2}}{d\text{pH}_s} \cdot \frac{d\text{pH}_s}{d\text{pH}_c} = 1.2658 \, (9.75 - \text{pH}_s)$$

$$\frac{1}{C_{\text{Hb}}} \cdot \frac{dC_{\text{BHbO}_2}}{d\text{pH}_c} = 1.2658 \ (9.75 + 1.6202 - 1.2658 \ \text{pH}_c)$$

$$= 1.2658 \ (11.3702 - 1.2658 \ \text{pH}_c)$$

$$= 14.39 - 1.6 \ \text{pH}_c$$
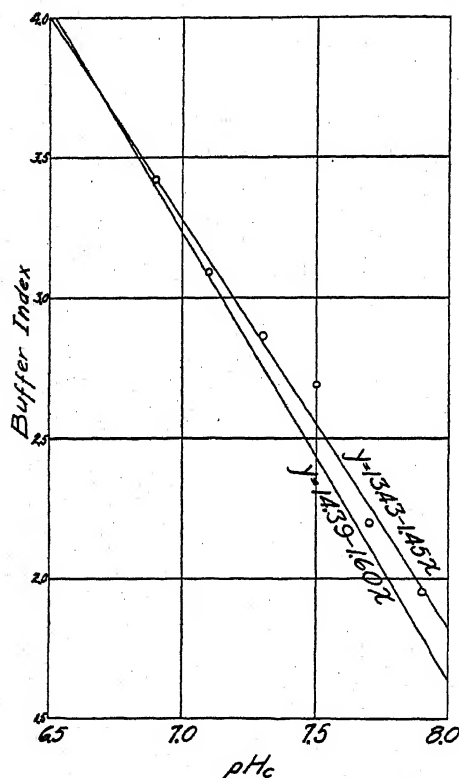
FIG. 3. The buffer index *referred to pH_c* of oxygenated red blood cells (lower curve) and of horse hemoglobin (upper curve).

The graph of this curve is shown on Fig. 3 together with points representing the mean values of the buffer index of horse oxy-hemoglobin of Table XVII, p. 130 of the paper of Hastings, Van Slyke, Neill, Heidelberger, and Harington (2). For convenience of comparison the latter values have been fitted with a straight line, $\frac{1}{C_{\text{Hb}}} \cdot \frac{C_{\text{BHbO}_2}}{d\text{pH}_c} = 13.43 - 1.45\text{pH}_c$. The agreement is ex-

cellent. The facts may be summed up by the statement that the buffer index of oxygenated red blood cells in the physiological range in man is approximately 0.1 less than that of horse hemoglobin. On account of the presence in the red blood cells of other buffer substances the difference between the two proteins may be assumed to be a little greater than this.

Fig. 4 shows the titration curves of oxygenated human red blood cells as determined by us and of horse oxyhemoglobin as deter-
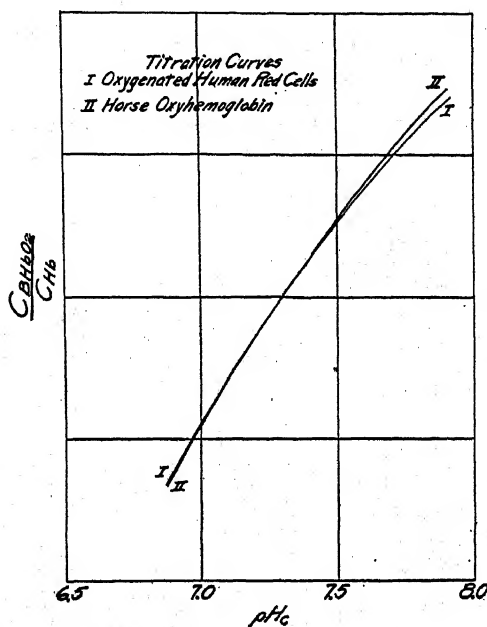
Fig. 4. Titration curves of oxygenated human red blood cells and of horse hemoglobin.

mined by Hastings, Van Slyke, Neill, Heidelberger, and Harington (2).

Their results are summed up in Table I, Columns 7 and 8.

III

During recent years evidence has been accumulating in this laboratory which seems to show that the approximation of Van

Slyke, Wu, and McLean (3) to a value of the function $\dfrac{d(V_c)}{d(pH_s)}$, $i.e.$
rate of change of cell volume with change of pH_s, ordinarily errs
slightly in excess of values experimentally observed.   We have
accordingly determined the value of this function in a large number
of instances.

Our latest and probably most accurate experiments have been
made upon five specimens of human blood.   The general char-
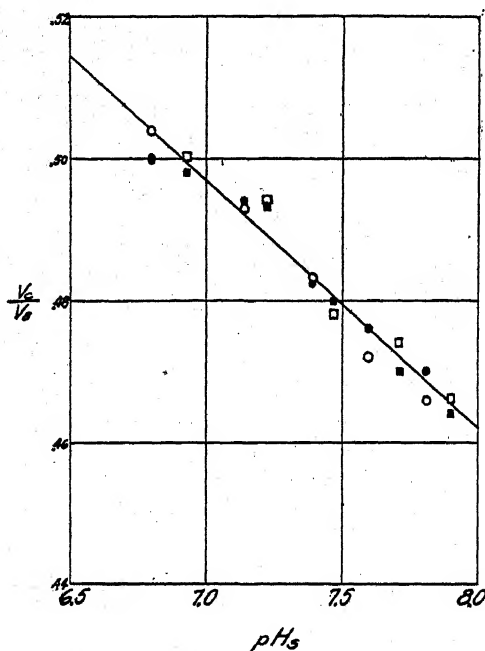
FIG. 5. $\dfrac{V_c^!}{V_B}$ as a function of pH_s

acter of the results will be sufficiently apparent from an examina-
tion of Figs. 5 and 6, which give in graphical form all experimen-
tally determined values of V_c as a function of pH_s of oxygenated
human blood in what appear to be the best and the worst experi-
ments of this last series.   The points represented have been
obtained in four different ways: (1) from hematocrit measure-
ments on oxygenated blood (circles), (2) from measurements of

the index of refraction of oxygenated blood (solid circles), (3) from hematocrit measurements of reduced blood (squares), and (4) from measurements of the index of refraction of reduced blood (filled in squares).   In cases (3) and (4) the V_c values for reduced blood have been converted into V_c values for oxygenated blood by means of the relation

$$V_c \text{ (oxygenated blood)} = V_c \text{ (reduced blood)} - y$$
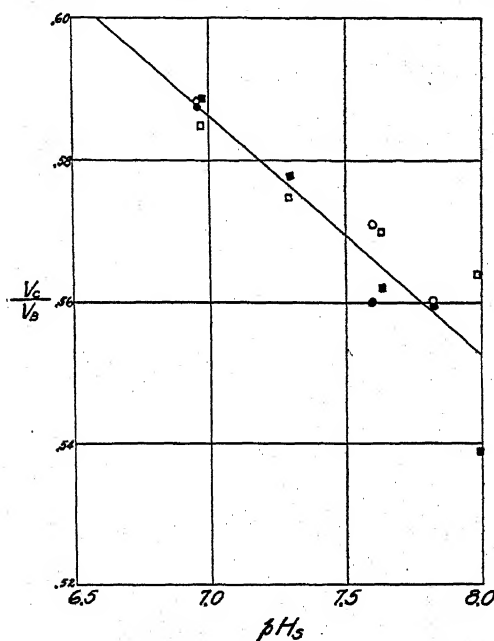
FIG. 6. $\dfrac{V_c}{V_B}$ as a function of pH$_s$

where $y = 0.28\text{pH}_s - 1.51$, an empirical result which seems to hold with fair accuracy for normal human blood when cell volume and serum volume are about equal.

These and all our other measurements seem to show, agreeably to the theory of Van Slyke, Wu, and McLean, that in general, for all specimens of blood, V_c is approximately a linear function of pH$_s$, i.e. $\dfrac{d(V_c)}{d(\text{pH}_s)} = $ constant for a given specimen of blood.   But

with variation in hemoglobin content of blood this function must become a variable.

It is evident that for Hb = 0 mM (plasma alone) and for Hb = 20 mM (cells alone for standard concentration of hemoglobin in cells) the value of $\dfrac{d(V_c)}{d(pH_s)}$ must be 0 and also that for some value of (Hb) such that cell water and serum water are approximately equal the value of $-\dfrac{d(V_c)}{d(pH_s)}$ may be expected to be a maximum. Our data for human blood fit approximately the parabola

$$\frac{d(V_c)}{d(pH_s)} = 0.14\,x^2 - 0.14\,x$$

where $x = \dfrac{\text{cell water}}{\text{blood water}}$ when $pH_s = 7.4$.   Here $-\dfrac{d(V_c)}{d(pH_s)}$ is a maximum when $x = 0.5$.   For standard concentrations of cells and plasma we have approximately, when $pH_s = 7.4$,

$$\frac{\text{Cell water}}{\text{Blood water}} = \frac{V_c}{1.3 - 0.3\,V_c} = \frac{(Hb)}{26 - 0.3\,(Hb)}$$

Fig. 7 gives the graph of $\dfrac{d(V_c)}{d(pH_s)} = f(Hb)$ obtained from the above relations and experimentally determined points.   With the exception of the five experiments on human blood already discussed, all points have been obtained exclusively from hematocrit measurements of cell volume in oxygenated blood.   In view of the smallness of the variation of $\dfrac{d(V_c)}{d(pH_s)}$ and the experimental errors that are as yet unavoidable, all points, except those for ox blood, fit the curve satisfactorily.   The experiments on ox blood were, however, the first of the present series and are perhaps less accurate than the others.   The significance of this discrepancy is therefore not clear.

Our experiments seem to show that the value of the function $\dfrac{d(V_c)}{d(pH_s)}$ for oxygenated blood is about three-fourths that estimated as a first approximation by Van Slyke, Wu, and McLean.   It should be noted that this implies very small differences, much

less than 0.01, in the ordinary measurements of the quantity $\dfrac{\text{cell volume}}{\text{blood volume}}$ and that such errors are usually negligible.
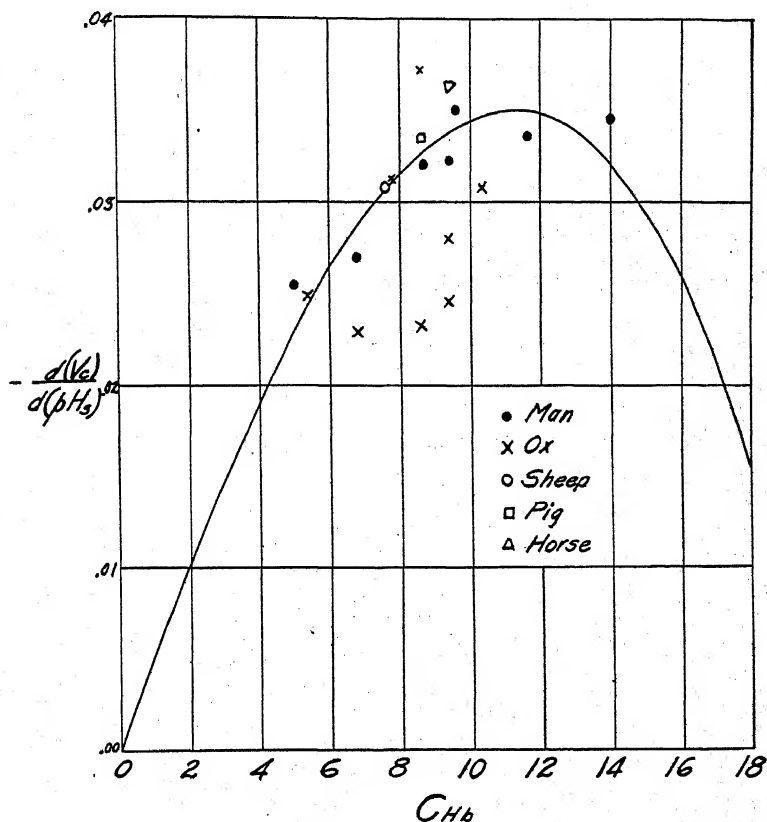
FIG. 7. — $\dfrac{dV_c}{d\mathrm{pH}_s}$ as a function of C_{Hb}

In view of the usefulness of Van Slyke, Wu, and McLean's estimate in the form in which they have given it, a revision of their figure, applying only to oxygenated blood, is given in Fig. 8.

The results of this section are summed up in Table II.

Some of the data presented in this section were taken from W. O. P. Morgan's thesis for the degree of Doctor of Philosophy in Cambridge University.
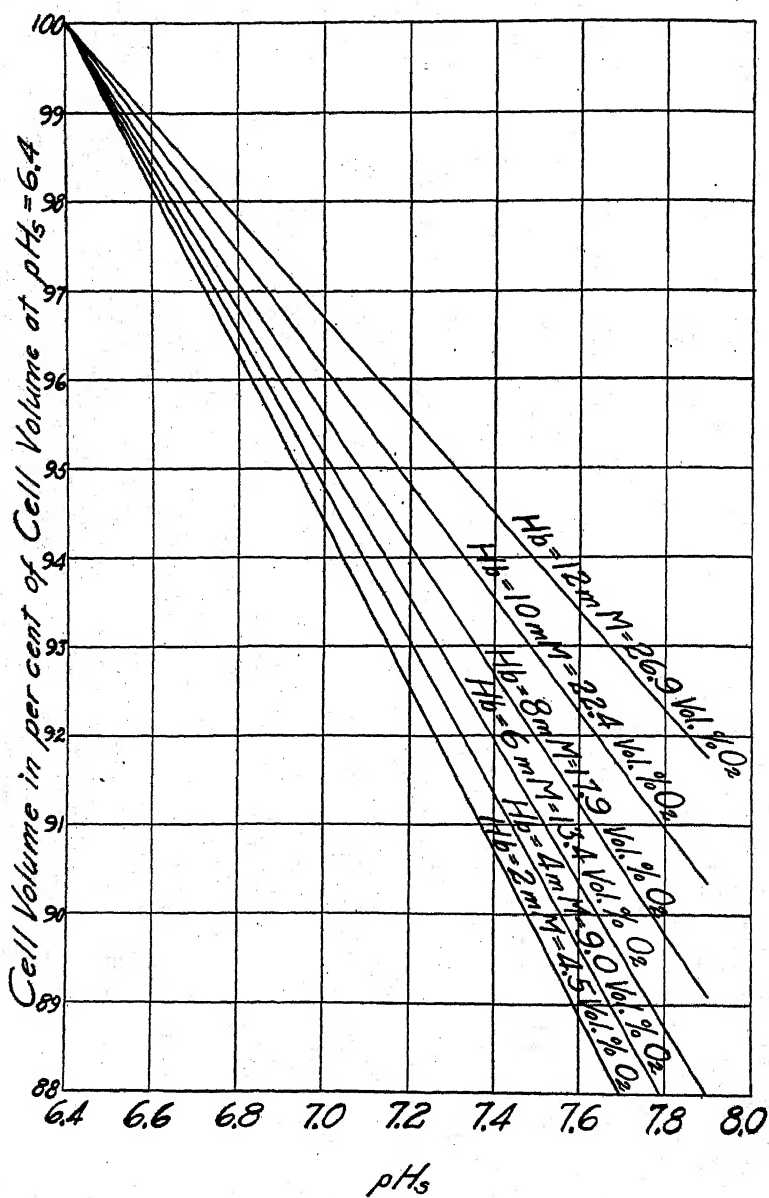
FIG. 8. Cell volume in per cent of cell volume at $pH_s = 6.4$ as a function of pH_s.

IV

The subject of this section is the distribution of combined carbonic acid (designated by $BHCO_3$) between cells and plasma in oxygenated human blood.

On Fig. 9 the results of experiments on six specimens of blood are shown. Abscissas are values of pH_s, ordinates values of the
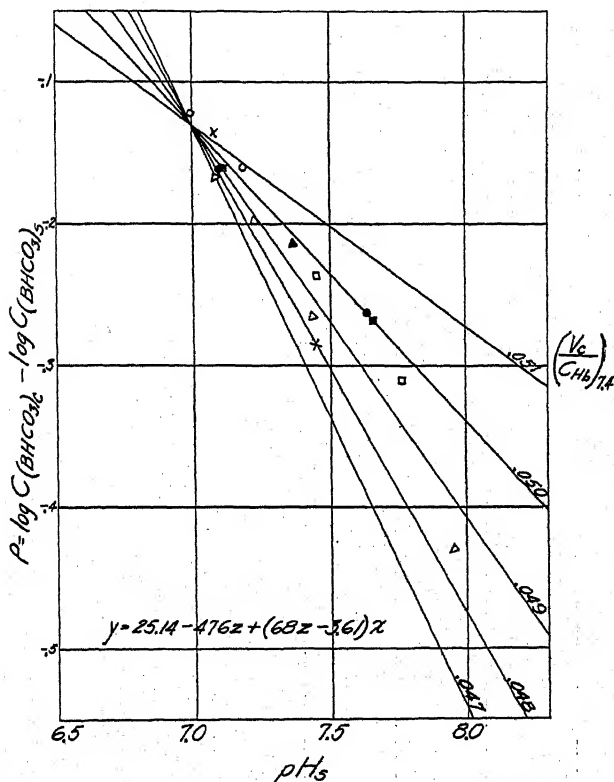
FIG. 9. Distribution of combined carbonic acid between cells and plasma of oxygenated human blood.

logarithm of the ratio of concentration of combined carbonic acid per liter of cells to concentration of combined carbonic acid per liter of serum. It is evident that the points are widely scattered.

In a first approximation this scattering may be attributed to

variation in concentration of hemoglobin in cells.   This is shown

in Fig. 10.   Here values of $\dfrac{\Delta\,(\log C_{(\mathrm{BHCO_3})_c} - \log C_{(\mathrm{BHCO_3})_s})}{\Delta\,\mathrm{pH}_s}$ are
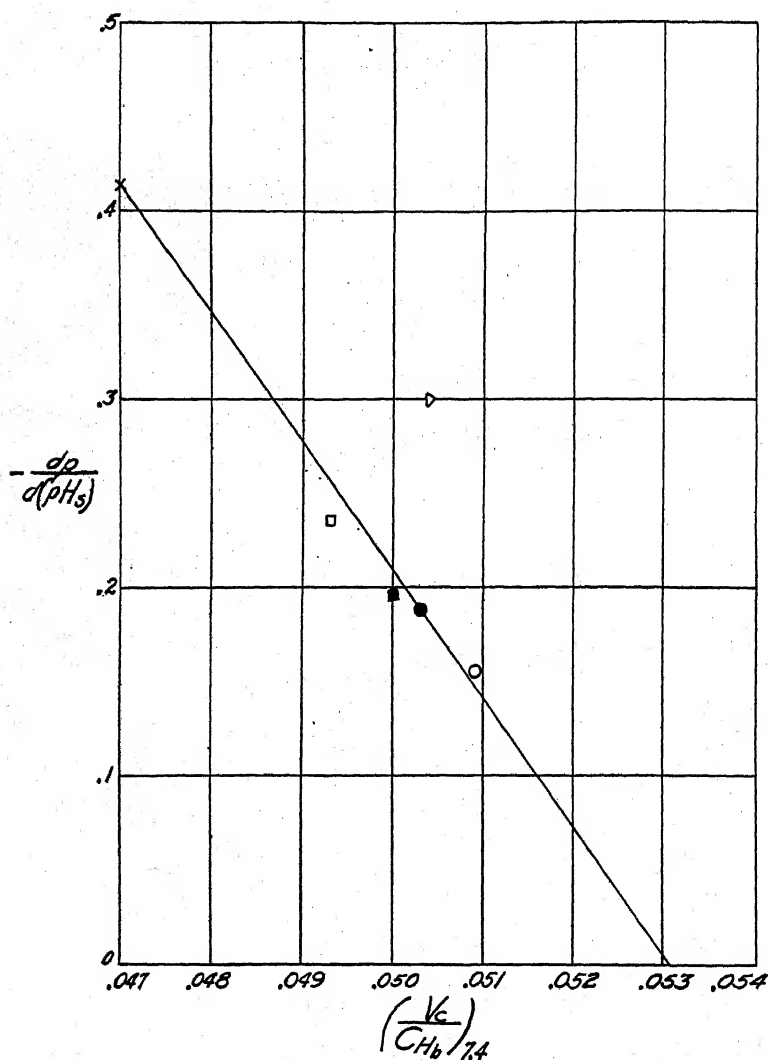
Fig. 10. $- \dfrac{\mathrm{dp}}{\mathrm{d(pH}_s)}$ as a function of $\left(\dfrac{V_c}{C_{\mathrm{Hb}}}\right)$ at pH = 7.4.   p = log $C_{(\mathrm{BHCO_3})_c} - \log C_{(\mathrm{BHCO_3})_s}.$

represented as a function of $\left(\dfrac{V_c}{\text{Hb}}\right)_{7.4}$, *i.e.* cell volume per m_M of hemoglobin when $pH_s = 7.4$, or in other words the dilution of hemoglobin in the cell.   With one exception there is close approximation to the relation

$$\frac{d\left(\log C_{(\text{BHCO}_3)_c} - \log C_{(\text{BHCO}_3)_s}\right)}{dpH_s} = 68\left(\frac{V_c}{\text{Hb}}\right)_{7.4} - 3.61 \qquad (1)$$

Inspection of Fig. 9 shows that at $pH_s = 7.0$ there is a marked tendency for the values of the ordinate to converge on the value -0.13.   This permits an estimate of the constant of integration of the above expression and leads to the equation

$$\text{Log}\,\frac{C_{(\text{BHCO}_3)_c}}{C_{(\text{BHCO}_3)_s}} = 25.14 - 476\left(\frac{V_c}{\text{Hb}}\right)_{7.4} + \left[68\left(\frac{V_c}{\text{Hb}}\right)_{7.4} - 3.61\right]pH_s \quad (2)$$

The contour lines of Fig. 9 are the geometrical expression of this equation.

It is hardly necessary to call attention to the uncertainty of this result for a wide range of values of the dilution of hemoglobin in cells.   However, for the standard dilution of hemoglobin in cells, $\left(\dfrac{V_c}{\text{Hb}}\right)_{7.4} = 0.050$, adopted in this paper, the equation reduces to the form

$$\text{Log}\,\frac{C_{(\text{BHCO}_3)_c}}{C_{(\text{BHCO}_3)_s}} = 1.34 - 0.21\,pH_s \qquad (3)$$

and for the validity of this expression we have confirmatory evidence in the results of studies of ten other specimens of blood. For these the mean value, at $pH_s = 7.45$, of $\log \dfrac{C_{(\text{BHCO}_3)_c}}{C_{(\text{BHCO}_3)_s}}$ was -0.237 and the mean value of $\left(\dfrac{V_c}{\text{Hb}}\right)_{7.4}$ was 0.0497, which is sufficiently close to the standard value of 0.050 to permit a satisfactory comparison.   The value of $\log \dfrac{C_{(\text{BHCO}_3)_c}}{C_{(\text{BHCO}_3)_s}}$ for $pH_s = 7.45$, cal-

culated from equation (2) is -0.233 and the value of pH_s for log $\dfrac{C_{(BHCO_3)_c}}{C_{(BHCO_3)_s}} = -0.237$ is 0.0496.   The agreement is within the limits of experimental error.   Accordingly it seems probable that equation (3) holds with fair accuracy for blood of standard concentrations of hemoglobin in cells.   It may be expected to fall short of precision when the concentrations of plasma proteins, of chlorides, and possibly of other substances vary widely from the normal.   Also it is not improbable that the relation cannot be regarded as approximately linear for extreme variations of pH_s.

From equation (3) other useful relations may be deduced.

Given $pH_s = 6.10 + \log C_{(BHCO_3)_s} - \log (0.0310\ pCO_2)$

and $pH_c = 5.97 + \log C_{(BHCO_3)_c} - \log (0.0264\ pCO_2)$

we have $pH_c = \log C_{(BHCO_3)_c} - \log C_{(BHCO_3)_s} + pH_s - 0.06$

whence, with equation (3)

$$pH_c = 1.28 + 0.79\ pH_s \qquad (4)$$

Also   serum $BHCO_3 = C_{(BHCO_3)_s} \cdot (1 - V_c)$

Cell $BHCO_3 = C_{(BHCO_3)_c} \cdot V_c$

$_{(BHCO_3)_B}$ = serum $BHCO_3$ + cell $BHCO_3 = C_{(BHCO_3)_s} - \left(C_{(BHCO_3)_s} - C_{(BHCO_3)_c}\right)$

and $$\frac{C_{(BHCO_3)_B}}{C_{(BHCO_3)_S}} = 1 - \left(1 - \frac{C_{(BHCO_3)_c}}{C_{(BHCO_3)_s}}\right) V_c \qquad (5)$$

Further $\dfrac{\text{Serum } BHCO_3}{C_{(BHCO_3)_B}} = \dfrac{C_{(BHCO_3)_S}}{C_{(BHCO_3)_B}} (1 - V_c)$

whence $$\frac{\text{Serum } BHCO_3}{C_{(BHCO_3)_B}} = \frac{1 - V_c}{1 - \left(1 - \dfrac{C_{(BHCO_3)_c}}{C_{(BHCO_3)_s}}\right) V_c} \qquad (6)$$

By combining equations (5) and (6) with equation (3) $\dfrac{C_{(BHCO_3)_B}}{C_{(BHCO_3)_S}}$

and $\dfrac{\text{serum BHCO}_3}{C_{(BHCO_3)_B}}$ may be obtained as explicit functions of V_c and C_{H_s}.

$$\frac{C_{(BHCO_3)_B}}{C_{(BHCO_3)_S}} = 1 - \left(1 - 21.88\left[C_{H_s}^{0.21}\right]\right) V_c \tag{7}$$

$$\frac{\text{Serum BHCO}_3}{(BHCO_3)_B} = \frac{1 - V_c}{1 - \left(1 - 21.88\left[C_{H_s}^{0.21}\right]\right) V_c} \tag{8}$$

Finally, it is possible to express values of $\dfrac{C_{(BHCO_3)_B}}{p CO_2}$ as a function of V_c and C_{H_s}. We have equation (7)

$$C_{(BHCO_3)_s} = \frac{C_{(BHCO_3)_B}}{1 - \left(1 - 21.88\left[C_{H_s}^{0.21}\right]\right) V_c}$$

Also
$$(H)_S = \frac{7.95 \times 10^{-7} \times 0.031\ p CO_2}{C_{(BHCO_3)_S}}$$

whence

$$(H)_S = 7.95 \times 10^{-7} \times 0.031 \left(1 - V_c + 21.88\left[C_{H_s}^{0.21}\right] V_c\right) \times \frac{p CO_2}{C_{(BHCO_3)_B}}$$

$$10^7 \times (H)_S = \left(0.24645\ (1 - V_c) + 5.3923\left[C_{H_s}^{0.21}\right]\right) \frac{p CO_2}{C_{(BHCO_3)_B}}$$

and
$$\frac{C_{(BHCO_3)_B}}{p CO_2} = \frac{0.24645\ (1 - V_c) + 5.3923\left(C_{H_s}^{0.21}\right)}{(H)_S \times 10^7} \tag{9}$$

The principal results of this section are summed up in the tables; equation (3) in Table I, Columns 1 and 3, equation (4) in Table I, Columns 1 and 2, equation (7) in Table IV, equation (8) in Table V, and equation (9) in Table VI.

Column 9 of Table I gives values of r_{HCO_3}, the ratio of the concentration of cell bicarbonate in cell water to the concentration of serum bicarbonate in serum water.   The values have been calculated for a hemoglobin content in blood of 10 mM.   If we calculate the values for 2 mM and for 14 mM from equations of this section we find values of r slightly different from those tabulated, except for $pH_s = 7.4$.   For example for a hemoglobin content of 2 mM per liter we have, at $pH_s = 6.8$, $r = 1.0330$ and for a concentration of 14 mM of hemoglobin $r = 1.0400$.   At $pH_s = 8.0$ we have for 2 mM of hemoglobin per liter of blood $r = 0.6087$ and for 14 mM of hemoglobin per liter of blood $r = 0.6023$.   It is evident, however, that we are quite as well justified in assuming r to be independent of the hemoglobin content of blood as to assume independence between the ratio of the concentration of bicarbonate per liter of cells and the concentration of bicarbonate per liter of serum, on the one hand, and hemoglobin content of blood on the other.   Since these variations in the value of r are also negligible it is neither logically misleading nor actually inaccurate to employ the values of Column 9, Table I, for all concentrations, and to assume that such errors as may be present in this table are due to the inaccuracy of the experiments upon which the whole computation is based.

In the tables a large number of decimal places have been retained in order to facilitate interpolation.   Until corrections have been found for the neglected variables (concentration of hemoglobin in cells and so forth) pH values are to be regarded as significant only to ± 0.01, C_{BHCO_3} values only to ± 0.1 and so forth.

<div align="center">V</div>

This section comprises a discussion of the accompanying tables, Tables I to VII.   We may first consider Table VII, by means of which, values of C_{Hb} and a pair of values of pCO_2 and $C_{(BHCO_3)B}$ being given, the value of pH_s may be estimated.   This is probably the least accurate estimate that may be made from any of the tables because all the errors of experiment upon which their construction is based and all variations from standard concentrations of hemoglobin in cells, of proteins in plasma, and of chlorides may be expected to exert a relatively large effect upon the estimate.   In order to test the accuracy of Table VII a comparison is given

in Table VIII of the values of pH$_s$ experimentally determined and estimated from Table VII for fifteen equilibrations of six specimens of blood.

TABLE I

Acid-Base Properties of Human Blood

pH$_s$ (1)	pH$_c$ (2)	$\log \frac{C(\text{BHCO}_3)_s}{C(\text{BHCO}_3)_c}$ (3)	$\frac{C(\text{BHCO}_3)_s}{C(\text{BHCO}_3)_c}$ (4)	$\frac{C_{\text{BP}_s}}{C_{\text{P}_s}}$ (5)	$\frac{1}{C_{\text{P}_s}} \cdot \frac{d(C_{\text{BP}_s})}{d(\text{pH}_s)}$ (6)	$\frac{C_{\text{BHbO}_2}}{C_{\text{Hb}}}$ (7)	$\frac{1}{C_{\text{Hb}}} \cdot \frac{d(C_{\text{BHbO}_2})}{d(\text{pH}_s)}$ (8)	r_{HCO_3} (9)
6.80	6.6520	0.0880	1.225	0.0000		0.0000	2.95	1.037
6.85	6.6915	0.0985	1.255	0.0045	0.092	0.1463	2.90	1.014
6.90	6.7310	0.1090	1.285	0.0092	0.096	0.2900	2.85	0.992
6.95	6.7705	0.1195	1.317	0.0141	0.100	0.4313	2.80	0.970
7.00	6.8100	0.1300	1.349	0.0192	0.103	0.5700	2.75	0.948
7.05	6.8495	0.1405	1.382	0.0244	0.106	0.7063	2.70	0.927
7.10	6.8890	0.1510	1.416	0.0298	0.110	0.8400	2.65	0.906
7.15	6.9285	0.1615	1.450	0.0354	0.114	0.9713	2.60	0.886
7.20	6.9680	0.1720	1.486	0.0412	0.116	1.1000	2.55	0.866
7.25	7.0075	0.1825	1.522	0.0470	0.116	1.2263	2.50	0.847
7.30	7.0470	0.1930	1.560	0.0528	0.115	1.3500	2.45	0.828
7.35	7.0865	0.2035	1.598	0.0585	0.113	1.4713	2.40	0.810
7.40	7.1260	0.2140	1.637	0.0641	0.111	1.5900	2.35	0.792
7.45	7.1655	0.2245	1.677	0.0696	0.110	1.7063	2.30	0.774
7.50	7.2050	0.2350	1.718	0.0751	0.109	1.8200	2.25	0.757
7.55	7.2445	0.2455	1.760	0.0805	0.107	1.9313	2.20	0.740
7.60	7.2840	0.2560	1.803	0.0858	0.105	2.0400	2.15	0.724
7.65	7.3235	0.2665	1.847	0.0910	0.104	2.1463	2.10	0.708
7.70	7.3630	0.2770	1.892	0.0962	0.103	2.2500	2.05	0.692
7.75	7.4025	0.2875	1.939	0.1013	0.101	2.3513	2.00	0.677
7.80	7.4420	0.2980	1.986	0.1063	0.100	2.4500	1.95	0.662
7.85	7.4815	0.3085	2.035	0.1113	0.100	2.5463	1.90	0.647
7.90	7.5210	0.3190	2.084	0.1163	0.100	2.6400	1.85	0.633
7.95	7.5605	0.3295	2.135	0.1213	0.100	2.7313	1.80	0.619
8.00	7.6000	0.3400	2.188	0.1263	0.100	2.8200	1.75	0.605

For three specimens of blood the errors in the estimate of pH$_s$ fall in absolute value below 0.01, for three other specimens their absolute values are, at least in some instances, larger. In two of the latter cases the error may be attributed to a wide departure

from the standard value 0.050 in the quantity $\left(\dfrac{V_c}{C_{\text{Hb}}}\right)$, in the third case it is perhaps due to variation from standard in concentration of plasma proteins or of chlorides.   On the whole then Table VII may be expected to give values of pH_s that are fairly trustworthy.

TABLE II

Values of $10,000 \, \Delta V_c$ $(\Delta V_c = V_c$ *When* $pH_s = x - V_c$ *When* $pH_s = 7.4)$

pH_s	$V_c = 0.1$	$V_c = 0.2$	$V_c = 0.3$	$V_c = 0.4$	$V_c = 0.5$	$V_c = 0.6$	$V_c = 0.7$
6.80	58	108	151	184	203	209	197
6.85	53	99	139	169	187	192	180
6.90	49	91	127	153	170	174	164
6.95	44	82	114	138	153	157	147
7.00	39	73	101	123	136	139	131
7.05	35	64	89	108	119	122	114
7.10	30	55	76	93	102	104	98
7.15	25	46	64	78	86	87	81
7.20	20	37	51	62	69	70	65
7.25	15	28	39	47	52	52	48
7.30	10	19	26	31	34	35	32
7.35	5	9	13	16	17	17	16
7.40	0	0	0	0	0	0	0
7.45	−5	−9	−13	−16	−17	−17	−16
7.50	−10	−19	−26	−31	−34	−35	−32
7.55	−15	−28	−39	−47	−52	−52	−48
7.60	−20	−38	−53	−63	−69	−70	−64
7.65	−26	−48	−66	−79	−86	−87	−80
7.70	−31	−58	−79	−95	−103	−104	−95
7.75	−37	−68	−93	−111	−120	−121	−111
7.80	−42	−78	−106	−127	−138	−138	−126
7.85	−48	−88	−120	−143	−156	−155	−142
7.90	−53	−98	−134	−160	−173	−172	−157
7.95	−59	−108	−149	−177	−190	−189	−172
8.00	−64	−119	−163	−193	−208	−206	−187

Example to illustrate use of table: If the value of V_c when $pH_s = 7.7$ is 0.400, $10,000 \, \Delta V_c = -95$; $\Delta V_c = -0.0095$, and for this specimen of blood the value of V_c when $pH_s = 7.4$ is $0.4000 + 0.0095 = 0.4095$.

Often, however, it is of secondary importance to know the absolute value of pH_s precisely.   What is of first import is a precise estimate of the variation of this function from one state of equilibration to another.   This error is here estimated by the

differences, for the same specimen of blood, between successive errors of pH_s. It will be seen that all but two of these values are in absolute magnitude less than 0.01. The largest of these errors, however, concerns a specimen of blood which is very exceptional in respect to concentration of hemoglobin in cells and the next a specimen for which one experimental measurement is open to

TABLE III

Values of 10,000 ΔV_c

pH_s	Hb = 2	Hb = 4	Hb = 6	Hb = 8	Hb = 10	Hb = 12	Hb = 14
6 80	61	114	157	188	206	209	193
6.85	56	104	144	173	189	192	177
6.90	51	95	131	157	172	174	161
6.95	46	85	117	141	155	157	145
7.00	41	76	104	125	138	139	129
7.05	36	66	91	110	120	122	113
7.10	30	57	78	94	103	104	97
7.15	25	47	65	78	86	87	80
7.20	20	38	52	63	69	70	64
7.25	15	28	39	47	52	52	48
7.30	10	19	26	31	34	35	32
7.35	5	9	13	16	17	17	16
7.40	0	0	0	0	0	0	0
7.45	−5	−9	−13	−16	−17	−17	−16
7.50	−10	−19	−26	−31	−34	−35	−32
7.55	−15	−28	−39	−47	−52	−52	−48
7.60	−20	−38	−52	−63	−69	−70	−64
7.65	−25	−47	−65	−78	−86	−87	−80
7.70	−30	−57	−78	−94	−103	−104	−97
7.75	−36	−66	−91	−110	−120	−122	−113
7.80	−41	−76	−104	−125	−138	−139	−129
7.85	−46	−85	−117	−141	−155	−157	−145
7.90	−51	−95	−131	−157	−172	−174	−161
7.95	−56	−104	−144	−173	−189	−192	−177
8.00	−61	−114	−157	−188	−206	−209	−193

question. Thus it seems probable that estimates of the variation of pH_s from one state of equilibration to another made by means of Table VII will ordinarily be satisfactory.

An independent test of the accuracy of the tables as a whole may be obtained by means of the data of the preceding paper of this series (4), giving values of C_{Hb} and total CO_2 of blood for

different values of pCO_2.   For all specimens of blood from normal individuals in this series of observations the differences in total CO_2 for different pressures of CO_2 (Δ total CO_2) as observed and as estimated from the tables have been compared.   We find a mean value of Δ total CO_2 calculated $-\Delta$ total CO_2 observed of $+0.1$

TABLE IV

$$Values\ of\ \frac{C_{(BHCO_3)B}}{C_{(BHCO_3)S}}$$

pH$_s$	$V_c = 0.1$	$V_c = 0.2$	$V_c = 0.3$	$V_c = 0.4$	$V_c = 0.5$	$V_c = 0.6$	$V_c = 0.7$
6.80	0.9817	0.9633	0.9450	0.9266	0.9083	0.8900	0.8716
6.85	0.9797	0.9594	0.9391	0.9188	0.8985	0.8783	0.8580
6.90	0.9778	0.9556	0.9334	0.9112	0.8890	0.8668	0.8446
6.95	0.9760	0.9519	0.9278	0.9038	0.8797	0.8557	0.8316
7.00	0.9741	0.9483	0.9224	0.8965	0.8707	0.8448	0.8189
7.05	0.9724	0.9447	0.9171	0.8894	0.8618	0.8342	0.8065
7.10	0.9706	0.9413	0.9119	0.8825	0.8532	0.8238	0.7944
7.15	0.9689	0.9379	0.9068	0.8758	0.8447	0.8137	0.7826
7.20	0.9673	0.9346	0.9019	0.8692	0.8365	0.8038	0.7711
7.25	0.9657	0.9314	0.8971	0.8628	0.8285	0.7941	0.7598
7.30	0.9641	0.9282	0.8924	0.8565	0.8206	0.7847	0.7489
7.35	0.9626	0.9252	0.8878	0.8504	0.8130	0.7755	0.7381
7.40	0.9611	0.9222	0.8833	0.8444	0.8055	0.7666	0.7277
7.45	0.9596	0.9193	0.8789	0.8385	0.7982	0.7578	0.7174
7.50	0.9582	0.9164	0.8746	0.8328	0.7911	0.7493	0.7075
7.55	0.9568	0.9136	0.8705	0.8273	0.7841	0.7409	0.6977
7.60	0.9555	0.9109	0.8664	0.8219	0.7773	0.7328	0.6882
7.65	0.9541	0.9083	0.8624	0.8166	0.7707	0.7248	0.6790
7.70	0.9528	0.9057	0.8585	0.8114	0.7642	0.7171	0.6699
7.75	0.9516	0.9032	0.8548	0.8063	0.7579	0.7095	0.6611
7.80	0.9504	0.9007	0.8511	0.8014	0.7518	0.7021	0.6525
7.85	0.9492	0.8983	0.8474	0.7966	0.7457	0.6949	0.6440
7.90	0.9480	0.8960	0.8439	0.7919	0.7399	0.6878	0.6358
7.95	0.9468	0.8937	0.8405	0.7873	0.7341	0.6810	0.6278
8.00	0.9457	0.8914	0.8371	0.7828	0.7285	0.6743	0.6200

MM.   In other words the mean error in estimating from the tables the difference in total CO_2 at two different pressures of CO_2 is $+$ 0.1 MM.   It seems probable that this small error is due to small mean variations from the standard concentration of hemoglobin in cells, of proteins in plasma, and of chlorides.   In other respects

the tables of this paper fit the data with approximately the precision obtained by the nomogram of the preceding paper (4).

An example will best illustrate one of the many uses of the tables. Given for a specimen of oxygenated blood, $C_{Hb} = 8.92$, $pCO_2 = 33.5$, and $C_{(BHCO_3)B} = 18.59$; required, the properties of

TABLE V

Values of $\dfrac{Serum\ Bicarbonate}{C_{(BHCO_3)B}}$

pH$_s$	$V_c = 0.1$	$V_c = 0.2$	$V_c = 0.3$	$V_c = 0.4$	$V_c = 0.5$	$V_c = 0.6$	$V_c = 0.7$
6.80	0.9168	0.8305	0.7408	0.6475	0.5505	0.4495	0.3442
6.85	0.9186	0.8338	0.7454	0.6530	0.5565	0.4555	0.3497
6.90	0.9204	0.8372	0.7499	0.6585	0.5624	0.4615	0.3552
6.95	0.9222	0.8404	0.7544	0.6639	0.5684	0.4675	0.3607
7.00	0.9239	0.8437	0.7589	0.6693	0.5743	0.4735	0.3663
7.05	0.9256	0.8468	0.7633	0.6746	0.5802	0.4795	0.3720
7.10	0.9272	0.8499	0.7676	0.6799	0.5861	0.4856	0.3776
7.15	0.9289	0.8530	0.7719	0.6851	0.5919	0.4916	0.3833
7.20	0.9304	0.8560	0.7762	0.6903	0.5977	0.4976	0.3891
7.25	0.9320	0.8589	0.7803	0.6954	0.6035	0.5037	0.3948
7.30	0.9335	0.8619	0.7844	0.7005	0.6093	0.5097	0.4006
7.35	0.9350	0.8647	0.7885	0.7056	0.6150	0.5158	0.4064
7.40	0.9364	0.8675	0.7925	0.7106	0.6208	0.5218	0.4123
7.45	0.9379	0.8703	0.7965	0.7155	0.6264	0.5278	0.4182
7.50	0.9393	0.8730	0.8003	0.7204	0.6321	0.5339	0.4241
7.55	0.9406	0.8756	0.8042	0.7253	0.6377	0.5399	0.4300
7.60	0.9420	0.8782	0.8080	0.7301	0.6432	0.5459	0.4359
7.65	0.9433	0.8808	0.8117	0.7348	0.6488	0.5519	0.4419
7.70	0.9445	0.8833	0.8154	0.7395	0.6543	0.5578	0.4478
7.75	0.9458	0.8858	0.8190	0.7441	0.6597	0.5638	0.4538
7.80	0.9470	0.8882	0.8225	0.7487	0.6651	0.5697	0.4598
7.85	0.9482	0.8906	0.8260	0.7532	0.6705	0.5756	0.4658
7.90	0.9494	0.8929	0.8295	0.7577	0.6758	0.5815	0.4718
7.95	0.9505	0.8952	0.8329	0.7621	0.6811	0.5874	0.4779
8.00	0.9517	0.8974	0.8362	0.7664	0.6863	0.5933	0.4839

the blood in this condition and also when the values of pH$_s$ are 7.000 and 7.800.

Table VII gives pH$_s$ = 7.436. Then the other values of the first column of Table IX are obtained directly from the other tables or indirectly by means of obvious computations. It should

be noted that $B = C_{(BHCO_3)B} + C_{(BHbO_2)} + C_{(BP_s)}$. The second and third columns are now readily obtained by using in turn the values 7.000 and 7.800 for pH_s and the value $B = 36.27$ obtained in forming the first column. In this manner an approximate description of all the physicochemical properties of the specimen

TABLE VI

Values of $\dfrac{C_{(BHCO_3)B}}{pCO_2}$

pH_s	$V_c = 0.1$	$V_c = 0.2$	$V_c = 0.3$	$V_c = 0.4$	$V_c = 0.5$	$V_c = 0.6$	$V_c = 0.7$
6.80	0.1525	0.1497	0.1469	0.1440	0.1412	0.1383	0.1355
6.85	0.1708	0.1673	0.1638	0.1602	0.1567	0.1532	0.1497
6.90	0.1913	0.1869	0.1826	0.1783	0.1740	0.1696	0.1653
6.95	0.2142	0.2089	0.2037	0.1984	0.1931	0.1879	0.1826
7.00	0.2399	0.2335	0.2272	0.2208	0.2145	0.2081	0.2018
7.05	0.2687	0.2611	0.2534	0.2458	0.2382	0.2306	0.2230
7.10	0.3009	0.2918	0.2828	0.2737	0.2646	0.2555	0.2464
7.15	0.3370	0.3263	0.3155	0.3047	0.2939	0.2832	0.2724
7.20	0.3775	0.3648	0.3521	0.3393	0.3266	0.3138	0.3011
7.25	0.4229	0.4079	0.3929	0.3779	0.3629	0.3479	0.3329
7.30	0.4737	0.4561	0.4385	0.4209	0.4033	0.3857	0.3681
7.35	0.5307	0.5101	0.4895	0.4689	0.4483	0.4277	0.4071
7.40	0.5945	0.5705	0.5464	0.5224	0.4984	0.4744	0.4503
7.45	0.6660	0.6381	0.6101	0.5821	0.5541	0.5262	0.4982
7.50	0.7462	0.7137	0.6812	0.6487	0.6162	0.5837	0.5512
7.55	0.8360	0.7983	0.7607	0.7230	0.6853	0.6476	0.6099
7.60	0.9367	0.8931	0.8495	0.8059	0.7623	0.7187	0.6750
7.65	1.0495	0.9991	0.9488	0.8984	0.8480	0.7976	0.7472
7.70	1.1760	1.1179	1.0597	1.0016	0.9435	0.8853	0.8272
7.75	1.3177	1.2508	1.1838	1.1168	1.0498	0.9829	0.9159
7.80	1.4766	1.3996	1.3225	1.2454	1.1684	1.0913	1.0142
7.85	1.6547	1.5661	1.4776	1.3890	1.3004	1.2119	1.1233
7.90	1.8543	1.7526	1.6509	1.5493	1.4476	1.3459	1.2443
7.95	2.0780	1.9614	1.8448	1.7282	1.6116	1.4950	1.3785
8.00	2.3288	2.1953	2.0617	1.9281	1.7945	1.6609	1.5274

when oxygenated, for all pressures of carbon dioxide, may be quickly obtained.

It is our purpose to endeavor to construct a similar set of tables for reduced human blood. With the two sets of tables it should be possible, when the value of C_{Hb} and one pair of values of pCO_2

and $C_{(BHCO_3)B}$ are given, to construct a complete nomogram for any specimen of human blood for standard concentrations of hemoglobin in cells, of proteins in plasma, and of chloride.   In view of the smallness of the errors attributable to variations in the values of these three independent variables it seems possible that they may ultimately be disposed of by means of three linear

TABLE VII

Values of pH_s

$\frac{(BHCO_3)_B}{pCO_2}$	Hb = 2	Hb = 4	Hb = 6	Hb = 8	Hb = 10	Hb = 12	Hb = 14
0.1	6.6134	6.6184	6.6236	6.6291	6.6349	6.6409	6.6474
0.2	6.9200	6.9310	6.9425	6.9548	6.9678	6.9818	6.9967
0.3	7.0989	7.1129	7.1278	7.1437	7.1609	7.1794	7.1995
0.4	7.2257	7.2416	7.2585	7.2767	7.2962	7.3175	7.3409
0.5	7.3238	7.3410	7.3594	7.3792	7.4007	7.4243	7.4505
0.6	7.4039	7.4221	7.4417	7.4629	7.4861	7.5116	7.5401
0.7	7.4716	7.4907	7.5114	7.5338	7.5583	7.5854	7.6155
0.8	7.5303	7.5502	7.5717	7.5951	7.6205	7.6485	7.6799
0.9	7.5820	7.6026	7.6246	7.6486	7.6748	7.7039	7.7368
1.0	7.6282	7.6492	7.6718	7.6964	7.7234	7.7534	7.7876
1.1	7.6700	7.6913	7.7144	7.7396	7.7673	7.7983	7.8336
1.2	7.7081	7.7298	7.7534	7.7790	7.8074	7.8392	7.8756
1.3	7.7431	7.7652	7.7892	7.8154	7.8443	7.8769	7.9142
1.4	7.7756	7.7980	7.8223	7.8490	7.8785	7.9118	7.9499
1.5	7.8058	7.8285	7.8532	7.8802	7.9103	7.9442	7.9832
1.6	7.8341	7.8570	7.8820	7.9095	7.9401	7.9746	8.0141
1.7	7.8606	7.8839	7.9092	7.9370	7.9680	8.0030	8.0428

corrections, involving no more than six further sets of tables.   If this can be done a complete quantitative description of the system, in so far as it may be regarded as constituted of seven components and two phases, will be attained.   It is known that specimens of human blood which cannot be so regarded in a close approximation are rare.

TABLE VIII

Test of Accuracy of Table VII

$\left(\frac{V_e}{C_{Hb}}\right)_{7.4}$	0.0493		0.0504			0.0470				0.0509		0.0503		0.0500	
Hb	8.92		8.47			9.86				9.24		9.29		10.19	
pCO_2	10.2	33.5	4.10	33.80	106.70	14.58	71.7	3.20	9.10	42.0	93.2	13.7	96.7	11.3	93.0
$C_{(BHCO_3)B}$	11.42	18.59	6.74	18.18	27.31	7.82	18.63	2.84	5.80	13.57	19.84	11.59	25.49	9.68	24.95
$\dfrac{C_{(BHCO_3)B}}{pCO_2}$	1.1196	0.5549	1.6439	0.5379	0.2560	0.5364	0.2598	0.8875	0.6374	0.3231	0.2129	0.8460	0.2636	0.8566	0.2683
pH, Observed	7.764	7.441	7.953	7.434	7.082	7.448	7.082	7.627	7.494	7.186	6.991	7.637	7.098	7.656	7.112
Calculated	7.760	7.436	7.929	7.418	7.070	7.431	7.089	7.657	7.505	7.188	6.990	7.637	7.092	7.654	7.107
Error pH$_s$	−0.004	−0.005	−0.024	−0.016	−0.012	−0.017	0.007	0.030	0.011	0.002	−0.001	0.000	−0.006	−0.002	−0.005
Δ error pH$_s$		0.001		−0.008	−0.004		−0.024	0.019	0.009		0.003		0.006		0.003

TABLE IX

Example of the Use of Preceding Tables

pH_s	7.436	7.000	7.800
pH_c	7.154	6.810	7.442
$\text{Log } \dfrac{C_{(BHCO_3)}}{C_{(BHCO_3)_c}}$	0.2216	0.1300	0.2980
$\dfrac{C_{(BHCO_3)_s}}{C_{(BHCO_3)_c}}$	1.666	1.349	1.986
$\dfrac{C_{BP_s}}{C_{P_s}}$	0.0681	0.0192	0.1063
$\dfrac{1}{C_{P_s}}\dfrac{dC_{BP_s}}{dpH_s}$	0.110	0.103	0.100
$\dfrac{C_{BHbO_2}}{C_{Hb}}$	1.6737	0.5700	2.45
$\dfrac{1}{C_{Hb}}\cdot\dfrac{dBHbO_2}{dpH_s}$	2.31	2.75	1.95
r_{HCO_3}	0.779	0.948	0.662
ΔV_c	-0.0016	0.0132	-0.0132
$(V_c)_{7.4}$	0.4460	0.4460	0.4460
V_c	0.4444	0.4592	0.4328
P_s	40.4	40.4	40.4
BP_s	2.751	0.776	4.295
$\dfrac{dC_{BP_s}}{dC_{pH_s}}$	4.44	4.16	4.04
C_{BHbO_2}	14.929	5.084	21.854
$\dfrac{dC_{BHbO_2}}{dpH_s}$	20.61	24.53	17.39
C_{BP_B}	17.68	5.86	26.15
$\dfrac{dC_{BP_B}}{dpH_s}$	25.05	28.69	21.43
B	36.27	36.27	36.27
$\dfrac{\text{Serum } BHCO_3}{C_{(BHCO_3)_B}}$	0.6756	0.6137	0.7224
Serum $BHCO_3$	12.56	18.66	7.30
Cell $BHCO_3$	6.03	11.75	2.81
$\dfrac{C_{(BHCO_3)_B}}{pCO_2}$	0.5549	0.2171	1.2201
pCO_2	33.5	140	8.3
$C_{(BHCO_3)_B}$	18.59	30.41	10.11
$C_{(H_2CO_3)_B}$	0.97	4.05	0.24
$(\text{Total } CO_2)_B$	19.56	34.46	10.35

SUMMARY

This paper presents in tabular form a quantitative description of oxygenated human blood of standard concentration of hemoglobin in cells, of proteins in plasma, and of chloride. The variables fully accounted for are oxygen capacity, base (so far as it is involved in the changing acid-base equilibrium), and pressure of carbon dioxide.

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THE USE OF COPPER AND IRON SALTS FOR THE DEPROTEINIZATION OF BLOOD

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As reported in a preliminary note (1), copper and iron salts were found to be suitable for the precipitation of blood proteins by the use of the simple technique followed in our method with zinc salts (2). Below we briefly describe the procedures and results obtained with these metals.

Copper

If to laked blood 1 volume of 10 per cent copper sulfate and 1 volume of 0.5 N sodium hydroxide are added, both proteins and non-sugar reducing substances are precipitated, leaving sugar as the sole reducing substance in the protein-free filtrate. Some copper always passes into the filtrate but this does not interfere with the determination of sugar. In cases of hyperglycemia, however, some sugar is lost in the process of precipitation in spite of the fact that the hydrogen ion concentration of the mixture (pH below 6) does not warrant the formation of insoluble copper hydroxide-sugar complexes. The probable explanation of the loss is the formation in the process of neutralization with sodium hydroxide of transitory alkaline strata in which sugar is precipitated with copper hydroxide and is not fully redissolved even though the ultimate reaction of the mixture is distinctly acid.

In order to overcome this difficulty we sought for a very weakly alkaline reagent to replace sodium hydroxide in the neutralization of the copper sulfate. Sodium tungstate proved to serve the purpose excellently. It precipitates copper at a lower pH than does sodium hydroxide, never renders the alkalinity of the mixture high enough to allow the formation of copper-sugar complexes, and at the same time is a very efficacious buffer at the slightly acid reaction optimal for the complete precipitation of blood proteins.

The reagents for the deproteinization of whole blood are as follows:

Solution I—7 per cent solution of $CuSO_4 \cdot 5H_2O$.

Solution II—10 per cent solution of $Na_2WO_4 \cdot 2H_2O$.

Procedure—To prepare blood filtrates in 1:10 dilution, lake 1 volume of blood in 7 volumes of water, add 1 volume of Solution I, and mix. Then introduce with continuous shaking 1 volume of Solution II, stopper the flask, shake well, and filter through dry filter paper after a few minutes. The filtrate is as a rule practically colorless since the excess copper is precipitated in the form of tungstate.

For the *deproteinization of corpuscles* the reagents must be used in concentrations twice as high as for whole blood. This does not necessitate the preparation of separate solutions, instead the corpuscles are laked in 5 volumes of water, and 2 volumes of each of the above solutions are added. It is advisable to allow the laked corpuscles to stand for a minute or 2 with the copper sulfate before adding the tungstate. This permits the transformation of hemoglobin into acid hematin, and as a consequence filtration is accelerated and a better yield of filtrate is obtained.

For the *precipitation of plasma or serum proteins* copper is superior to zinc. We made the observation in the tungstate method of Folin and Wu that the optimal reaction for the complete precipitation of the proteins of separated plasma or serum lies at a slightly higher acidity than for the total proteins of whole blood. The same holds true for precipitation by zinc or copper. If, however, the acidity required for a perfect precipitation of plasma proteins is maintained in the zinc method, zinc salts pass into the filtrate in quantities that interfere with the sugar determination. This entails the added work of removing the zinc from the filtrate by precipitation with alkali and a second filtration. The use of copper instead of zinc obviates this operation, since the presence of copper in the protein-free filtrate does not affect in the least the determination of sugar with alkaline copper reagents. It also is superfluous to neutralize the filtrates if the alkaline copper reagent is an efficacious buffer solution as the modified Shaffer-Hartmann reagent. Accordingly, we use for the deproteinization of plasma or serum relatively less alkali (sodium tungstate) than for whole blood. The reagents securing a perfect precipitation of plasma proteins are as follows:

Solution I—5 per cent solution of $CuSO_4 \cdot 5H_2O$.
Solution II—6 per cent solution of $Na_2WO_4 \cdot 2H_2O$.

The procedure is the same as for whole blood.

Protein-free blood filtrates, prepared by the copper method, yield true sugar values. In Table I are presented examples of comparative determinations in copper filtrates, zinc filtrates, and tungstate filtrates of blood samples obtained from hospital patients. As can be seen, there is good agreement between the sugar values in copper and zinc filtrates, and both are well in line

TABLE I

Comparative Sugar Determinations Showing That Blood Filtrates Prepared by Deproteinization with Copper Yield True Sugar Values

Results are given per 100 cc. of blood, in terms of glucose.

Experiment No.	Copper precipitation	Zinc precipitation	Tungstate precipitation		
			Apparent sugar (total reduction)	Reducing non-sugars (non-fermentable)	True sugar (fermentable)
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
1	74	70	98	27	71
2	214	215	238	23	215
3	204	210	231	19	212
4	288	289	319	26	293
5	92	93	110	16	94
6	230	231	264	26	238
7	260	259	285	25	260
8	67	62	82	22	60
9	154	147	171	22	149
10	143	144	167	35	142

with the true sugar values (apparent sugar minus reducing non-sugars) in tungstate filtrates.

Comparative determinations of plasma sugar show in copper filtrates 5 to 10 mg. per cent lower values than in tungstate filtrates. The differences correspond to the amounts of non-sugar reducing substances occurring in the tungstate filtrates of blood plasma.

Iron

Iron was introduced as a precipitant of blood proteins by Michaelis and Rona about 20 years ago. In their method the iron

is added to the laked blood in the form of colloidal ferric hydroxide. We found that a complete precipitation is also effected by the more convenient and simple technique of adding the iron in the form of a ferric salt (preferably sulfate) and subsequent precipitation with alkali, as in our technique with zinc or copper salts.

The *reagents* for the procedure are an approximately 15 per cent solution of ferric sulfate (Merck's C.P. reagent was used) and $\frac{2}{3}$ N sodium hydroxide. The two reagents must be so balanced that when 2 cc. of the ferric sulfate solution are titrated with the alkali, 4.9 to 5.1 cc. are required to produce a permanent pink color with phenolphthalein. The ferric sulfate is diluted with about 50 cc. of water before titration. It is essential to run in the alkali slowly with continuous shaking.

Procedure—To prepare filtrates in 1:10 dilution, lake the blood in 6 volumes of water, introduce 1 volume of the ferric sulfate solution, mix, then add with continuous shaking 2 volumes of the alkali, stopper the flask, and shake well. Filter through dry filter paper a few minutes later.

The filtrate is clear, but has a faint yellow tinge due to the presence of a slight quantity of iron. This is inevitable, since a higher degree of alkalinity, which would suffice to remove the last traces of iron, prevents the complete precipitation of proteins. The iron, however, interferes with the sugar determination, in that it lowers the results by 2 to 4 mg. per cent, and, therefore, must be eliminated prior to the sugar determination. By the addition of a few granules of sodium carbonate to the filtrate, the iron is precipitated and may be removed either by filtration or by centrifugation. The resulting colorless fluid yields true sugar values well in line with those obtained upon precipitation by zinc or copper. Notwithstanding the complication arising from the presence of iron in the protein-free filtrate, the technique with iron *salts* is simpler than precipitation by colloidal ferric hydroxide, especially since the latter needs to be combined with heat coagulation to give good results (3).

SUMMARY

Iron salts can be used instead of colloidal iron hydroxide for the removal of blood proteins in the determination of true sugar.

Copper salts are preferable to iron salts for this purpose, and fully the equal of zinc salts in regard to speed and simplicity of technique. For the precipitation of plasma or serum proteins copper is superior to zinc.

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NOTE ON THE DISTRIBUTION OF BLOOD SUGAR

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A recent paper by Folin and Svedberg (1) introduces new perplexities in the problem of the distribution of blood sugar between corpuscles and plasma. Until recently the view that the concentration of sugar in corpuscles and in plasma is nearly the same found general acceptance. Expressed in a convenient term this means that the ratio of distribution, corpuscle sugar: plasma sugar, is about unity. In 1928 the writer, prompted by his observation that the amount of non-sugar substances which reduce alkaline copper solutions is about 5 times as much in corpuscles as in plasma (2), studied the problem with the determination of true sugar values, and reported that the corpuscles contain relatively less sugar than the plasma. The distribution ratios, observed on thirty-six healthy subjects, gave the average of 77:100 (3). Ege and Roche in 1930 fully corroborated these results, finding an average ratio of 80:100 (4). Folin and Svedberg now report the substantially lower ratio of 60:100 for healthy persons.

The divergencies are even more striking in regard to the blood of diabetic subjects. Investigators prior to 1928 contended that the distribution ratio is distinctly lower in diabetic than non-diabetic cases. (This was then accepted as "the fundamental fact" in the structure of various theories advanced by Loewi and his collaborators, by Falta and others in attempts to throw light upon the nature of diabetes mellitus.) Diametrically opposed to this stands the report of Folin and Svedberg (1) to the effect that the corpuscles in the blood of diabetics contain relatively more sugar than in the blood of non-diabetic subjects. Both of these views are in sharp contrast to our own experimental evidence reported in 1928, showing that the relative sugar content of corpuscles in diabetic and non-diabetic cases is the same. The aver-

age corpuscle sugar: plasma sugar ratio for seventeen diabetic cases was 77:100, identical with the figure we reported for the non-diabetic cases.

As regards the earlier investigators, we have shown, that since they determined total reduction values instead of the true sugar content of corpuscles and plasma, their results do not express the actual distribution of blood sugar. If this error is corrected by deducting the amount of non-sugar reducing substances from the apparent sugar values of both corpuscles and plasma, the entire picture is changed. First, the distribution ratio drops below unity in non-diabetic as well as in diabetic cases, and, second, the difference between diabetic and non-diabetic cases is wiped out (3, 5), so that the older results fall in line with our findings which are based upon true sugar values.

Comparison of Folin and Svedberg's results with ours, however, presents a more difficult situation, since these authors also claim that their figures represent the true relation between the sugar contents of corpuscles and plasma. In order to scrutinize the validity of our previous findings, we undertook an experimental reexamination of the problem. Such revision seemed the more desirable as we now possess, in the method of deproteinization with zinc or copper, a simple and more direct approach to the determination of true sugar than in our past work, where true sugar was obtained as the difference of two separately determined reduction values.

In the present work sugar was determined in whole blood after deproteinization with zinc (6), and in plasma after deproteinization with copper (7). In addition the cell volume was obtained for the calculation of corpuscle sugar. (As pointed out in our older work, corpuscle sugar values obtained by direct determination are worthless, since they are always too low because of loss by glycolysis during centrifugation.) In Table I are presented the results of fifteen experiments, the first nine samples obtained from healthy individuals, the remaining six from diabetic patients. The average of the distribution ratio for the fifteen cases is 0.79, with variations from 0.70 to 0.86 in the non-diabetic, and from 0.75 to 0.84 for the diabetic cases, figures that fully confirm our previous results. We believe it to be a fair proof of accuracy and correctness that two substantially different analytical procedures

(both carefully checked with all available criteria for adequacy) led to practically identical results.

Inspection of Folin and Svedberg's analytical data, on the other hand, reveals that these authors determined total reduction values and not the true sugar in plasma. Granting that their figures for whole blood represent true sugar values (this is possible if loss of

TABLE I

Distribution of Sugar between Corpuscles and Plasma in Human Blood

The figures represent true sugar values determined in zinc filtrates.

Experiment No.	Cell volume	Sugar per 100 cc. of			Ratio $\frac{corpuscle\ sugar}{plasma\ sugar}$
		Whole blood	Plasma	Corpuscles (calculated)	
	per cent	mg.	mg.	mg.	
1	42	99	106	89	0.84
2	45	98	108	86	0.80
3	45	111	125	94	0.75
4	44	92	106	75	0.71
5	48	114	122	105	0.86
6	48	117	132	101	0.77
7	47	129	147	109	0.74
8	36	103	111	89	0.80
9	50	137	160	114	0.70
10	43	182	196	163	0.83
11	44	233	253	207	0.82
12	44	228	246	205	0.83
13	48	262	285	239	0.84
14	47	367	408	321	0.79
15	36	405	445	334	0.75
Lowest					0.70
Highest					0.86
Average					0.79

sugar with the precipitated unlaked corpuscles approximately cancels the amount of non-sugar reducing substances derived from the plasma), the fact that for plasma total reduction values were substituted for true sugar still remains an appreciable source of error. We shall presently show that, slight and negligible as the amount of non-sugar reducing substances in plasma may appear (5 to 6 mg. per cent in equivalents of glucose as determined by the

Folin-Wu method),[1] it suffices grossly to distort the true picture of the distribution of sugar. The relations involved can be best demonstrated with Folin and Svedberg's own figures, as given in the accompanying tabular matter, by showing how the distribution ratio is affected when the plasma sugar values are corrected by the deduction of 5 mg. per cent of reducing non-sugars.

	Uncorrected mg. per cent	Corrected mg. per cent
First case of Table I (Adams)		
Sugar in unlaked whole blood..............	72	72
Plasma sugar.............................	86	81
Corpuscle sugar, calculated on basis of cell volume of 42 per cent....................	53	60
Ratio of distribution, corpuscle sugar : plasma sugar...................................	0.61	0.74
Case 7 in Table III		
Sugar in unlaked whole blood..............	73	73
Plasma sugar.............................	87	82
Cell volume 50 per cent, corpuscle sugar (calculated).................................	59	64
Ratio of distribution......................	0.68	0.78

It is evident from these examples that when the apparent plasma sugar values, as recorded in Folin and Svedberg's tables, are replaced by corrected values which are nearer true sugar values, the distribution ratio proves to be considerably higher than calculated by Folin and Svedberg. In fact, the results are well in accord with our own.

The two examples above are representative of normal cases with low blood sugar level. The following is the diabetic Case 1 in their Table III.

	Uncorrected mg. per cent	Corrected mg. per cent
Sugar in unlaked whole blood................	230	230
Plasma sugar...............................	263	258
Corpuscle sugar, calculated on basis of cell volume of 42 per cent......................	184	191
Ratio of distribution........................	0.69	0.74

[1] In a previous paper (this *Journal*, 80, 738 (1928)), Somogyi and Kramer reported a figure of 4 mg. of non-glucose reducing material per 100 cc. of plasma (four determinations). The average figure obtained from over a dozen such determinations is about 5 mg. per cent.

As can be seen, the correction of the plasma sugar entails in the diabetic case, as in the non-diabetic, a rise in the distribution ratio and at once removes the apparent difference between the distribution ratios of diabetic and non-diabetic cases. The difference is' actually non-existent, and its origin is explained by a simple arithmetical consideration. It can be seen in the above examples that the inclusion of the reducing non-sugar substances in the plasma sugar values lowers in both diabetic and non-diabetic cases the quotient representing the distribution of blood sugar. This effect is the more marked the lower the blood sugar level is, while at very high hyperglycemic levels it becomes almost negligible. As a result of this relation Folin and Svedberg obtain distribution ratios for the blood of healthy, fasting subjects that are much too low, and at the same time are led to the misconception that the ratio in the diabetic (hyperglycemic) cases is higher than in the non-diabetic.

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THE INDUCTION OF TETANY IN RACHITIC RATS BY MEANS OF A NORMAL DIET*

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For many years the common method of inducing experimental tetany has been the removal of the parathyroid glands. This operation generally has been carried out on dogs and the thyroid gland has been excised incidentally. Following this procedure the calcium concentration of the blood promptly falls and the animal develops convulsions or minor signs of nervous hyperexcitability. Although a great deal has been learned by means of this experimental technique, it is evident that, from a pathogenetic standpoint, this mechanism bears little analogy to clinical tetany. Another method which has been employed to induce experimental tetany is the feeding of large amounts of phosphates. This procedure likewise must be regarded as far from satisfactory as it does not resemble the dietary conditions which are associated with the development of tetany in human beings.

By far the most common form of tetany is that which occurs in infants. Infantile tetany comes about almost invariably as a sequel to rickets and it is therefore remarkable that investigators have not emphasized the desirability of inducing experimental rickets previous to the induction of experimental tetany. It should be added, however, that for some time Shohl and his associates (1, 2) have clearly recognized the importance of this preliminary step and have reported a number of investigations in which tetany was occasioned by giving phosphates to rachitic rats. More recently Hamilton and his coworkers (3) have in-

* Presented in abstract before the Society for Experimental Biology and Medicine, December 17, 1930.

duced tetany in rabbits which previously had been rendered rachitic.

Our object was to attempt to induce tetany in rachitic animals by means of a ration which approximated, as nearly as possible, the normal dietary of the infant. The preliminary step in this undertaking was to bring about rickets in young rats in the established way by feeding a ration high in calcium and low in phosphorus. For this purpose the standard McCollum ration[1] was used in which the Ca:P ratio is about 4:1. This ration was fed for about 21 days and typical rickets resulted. With the object of developing tetany, the animals were then given the Sherman Diet B normal dietary, which is composed of $\frac{1}{3}$ dry milk, and $\frac{2}{3}$ whole wheat with the addition of 1.3 per cent sodium chloride. Its ratio of Ca:P is about 0.67:1 and it is stated to be adequate for normal growth and nutrition. After a period of 1, 2, or 4 days it was found that the calcium concentration of the serum of the rachitic rats had fallen from about 10 to about 7 mg. and in turn that the inorganic phosphate had risen to 8 or 10 mg. per 100 cc. of serum. At this time some of the animals manifested definite tremor on etherization, which in some instances developed into convulsive seizures. In view of the adequacy of the diet, this result was surprising. It will be noted from the accompanying table (Table I) that the animals which had been given this ration for a period of 8 days were found to have normal concentrations of calcium and phosphorus; in other words that the regulatory mechanism of the body had by this time been able to restore normal conditions.

Next, we fed a ration which had a larger proportion of milk. This is termed the Sherman Diet D and contains $\frac{2}{3}$ dry milk with $\frac{1}{3}$ whole wheat and sodium chloride, the ratio of Ca:P being about 1:1. The result was the same—a sharp fall of calcium with an associated rise of phosphate in the blood. It will be noted (Table II) that a chemical change in the blood had come about even after this normal diet had been given for a period of only 8 hours. After this short interval although no fall in calcium was evident, there was a rise in the phosphate concentration. In this connection it should be observed that when the calcium and phosphorus re-

[1] See McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., *J. Biol. Chem.*, **47**, 507 (1921).

turned to normal after a feeding period of a week or more, it was generally found that the phosphate returned somewhat more slowly and later than the calcium. In the course of these various tests most of the animals did not lose weight so that the change in calcium and phosphorus could not be interpreted as a reaction

TABLE I

Tetany Induced by Changing from Rickets-Producing Diet (Ca:P = 4:1) to Normal Diet (Ca:P = 0.67)

Weights of rats	McCollum rickets-producing diet fed	Rickets shown by x-ray	Subsequent diet	Healing shown by x-ray	Serum	
					Ca	P
gm.	*days*					
72, 94, 92	27	Moderate	Sherman	Trace	8.2	10.1
80, 100, 92		"	Diet B*	"	8.6	13.1
90, 114, 108		"	(2 days)	"	9.0	13.0
70, 90, 80		"		"	6.6	
70, 84, 84	29	"	McCollum	None	10.9	4.1
90, 100, 88	27	"	Sherman	Marked	5.9	13.4
88, 110, 110	.	"	Diet B	"	8.7	8.2
80, 100, 94		"	(4 days)	Moderate	6.8	11.8
70, 90, 86		"		"	7.2	7.6
78, 100, 98		"		"	8.8	9.6
74, 100, 100	31	"	McCollum	None	11.5	1.7
80, 100, 100		"		"	12.1	2.8
70, 84, 100	27	"	Sherman	Marked	10.2	9.3
76, 110, 134		"	Diet B	"	11.7	9.1
86, 114, 140		"	(8 days)	"	11.7	9.0
70, 102, 120		"		"	10.8	9.8
80, 110, 110		"		"	9.4	9.9
70, 90, 90		"		"	8.8	8.0

* ⅓ dried whole milk, ⅔ whole wheat, 1.3 per cent NaCl.

to starvation. Table II shows that the rats, in which the calcium fell after an interval of 1 day, had gained about 10 gm. each. Radiographs, which were taken regularly at the end of the tests, showed definite healing of the epiphyses, even after an interval of 2 days or less. However this phenomenon is not essential.

It was soon found that the same striking reaction could be in-

duced in rachitic rats by feeding whole dried milk, which contains an excess of calcium over·phosphorus of 1.3:1 (Table III).   In order to make the dietary resemble that of infants even more closely,

TABLE II

Tetany Induced by Changing from Rickets-Producing Diet (Ca:P = 4:1) to Normal Diet (Ca:P = 1.01)

Weights of rats	McCollum rickets-producing diet fed	Rickets shown by x-ray	Subsequent diet	Healing shown by x-ray	Serum	
					Ca	P
gm.	*days*					
80, 98, 100	20	Moderate	Sherman	None	8.7	6.6
72, 88, 88		"	Diet D*	"	9.3	7.6
			(8 hrs).			
66, 84, 84	20	"	McCollum	"	9.8	3.0
50, 70, 80	19	"	Sherman	Slight	7.8†	13.5
50, 64, 74		"	Diet D	"	7.8	10.0
50, 70, 78		"	(1 day)	Trace	5.9†	10.9
48, 60, 72		"		Slight •	6.8	9.6
50, 70, 70	20	"	McCollum	None	10.9	4.2
54, 74, 64	22	"	Sherman	Trace	†	8.6
40, 70, 72		"	Diet D	Slight or moderate	6.5	10.0
40, 60, 60		"	(2 days)	" " "	5.6†	7.5
40, 62, 60		"		Trace		9.6
60, 68, 68	24	"	McCollum	None		2.9
64, 94, 88		"	.	"	10.9	2.5
88, 100, 104	22	"	Sherman	Moderate	8.4†	6.7
40, 80, 80		"	Diet D	"	7.9	
50, 64, 64		"	(4 days)	"	†	8.7
40, 50, 54		"		"	9.4†	10.2

* ⅔ dried whole milk, ⅓ whole wheat, 0.7 per cent NaCl.
† Tremors. .

1 gm. of carrots was added to the whole milk ration in some instances.   The fall of ·calcium was similar.   In some tests 6 or even 12 per cent of lactose was added to the ⅔ milk ration, as it

has been thought by some that lactose protects animals from tetany by bringing about an acid reaction in the intestinal tract. It was found, however, in these experiments that the lactose was without effect.

In order to ascertain whether the drop in calcium could be brought about when the calcium was present in still greater excess, calcium lactate to the amount of 2.9 per cent was added to the $\frac{2}{3}$ milk ration; this addition rendered the calcium approximately

TABLE III

Tetany Induced by Changing from Rickets-Producing Diet (Ca:P = 4:1) to Diet of Whole Milk (Ca:P = 1.33)

Weights of rats	McCollum rickets-producing diet fed	Rickets shown by x-ray	Subsequent diet	Healing shown by x-ray	Serum	
					Ca	P
gm.	*days*					
50, 80, 70	19	Moderate	Whole milk	Slight	6.7	
44, 60, 70		"	(dried)	"	8.2	14.6
50, 74, 64		"	(2 days)	Trace	6.8	
54, 76, 74		"		"	6.5	11.8
50, 70, 70	21	"	McCollum	None	10.9	4.2
50, 70, 62	19	"	Whole milk	Moderate	7.2	11.7
54, 60, 74		"	(4 days)	"		
50, 70, 80		"		Marked		
60, 80, 84		"		"	8.4	11.7
50, 64, 70	23	"	McCollum	None	10.6	3.1
50, 60, 68		"		"		

equivalent to the calcium content of whole milk, making the ratio about 1.7:1. The fall of calcium was irregular on this diet; in some animals it did not come about (Table IV). However, it was evident from this experiment that in spite of the fact that a ration contains considerably more calcium than phosphorus, the concentration of calcium in the blood may fall far below the normal level. In the next experiment the ratio of calcium to phosphorus was raised somewhat higher, 2.1:1, by the addition of 4.7 per cent of calcium lactate to the $\frac{2}{3}$ milk diet. This ratio was not suffi-

ciently low; the calcium in the blood no longer fell, although healing of the epiphyses was marked.

It was found that in order to effect a drop in calcium in this way it is necessary to induce well developed rickets in the experimental animals. If the rickets-producing diet was given for a period of only 7 days instead of the usual 21 days and the dietary then was changed to the $\frac{2}{3}$ milk ration, the fall in calcium did not ensue. Furthermore, the change in diet must be brought about suddenly.

TABLE IV

Failure to Induce Tetany by Changing from Rickets-Producing Diet (Ca:P = 4:1) to a Diet with a Ratio of Ca:P = 2.1:1

Weights of rats	Rickets-producing diet	Rickets shown by x-ray	Subsequent diet	Healing shown by x-ray	Serum	
					Ca	P
gm.						
40, 74, 70	McCollum	Moderate	Sherman Diet D	Marked	10.1	11.8
70, 84, 86	(Ca:P =	"	+ 2.9 per cent	"	7.0	10.7
60, 80, 94	4:1)	"	Ca lactate*	"	10.3	10.6
52, 64, 80		"	(Ca:P = 1.7:1)	Moderate	8.4	8.9
66, 80, 90	Same	"	Sherman Diet D	"	10.6	8.5
58, 76, 88		Slight	+ 4.7 per cent	"		
			Ca lactate			
			(Ca:P = 2.1:1)			
82, 104, 110	Same	Moderate	McCollum	None	10.9	3.1
66, 80, 86		"		"	10.8	4.3
60, 80, 86		"		"	10.4	3.9
68, 72, 80		"		"	10.3	4.3

* Ca was added to render it equivalent to the Ca in whole milk.

For example, if the change from the McCollum ration, in which the ratio of Ca: P is about 4:1, is accomplished by degrees, being changed on the 1st day to a diet having a ratio of 3:1, on the 2nd day to one of 2:1, and on the 3rd day to one in which the ratio is 1:1, the serum calcium maintains its level and no signs of tetany develop.

In the next series of tests the ratio of 4:1 was maintained, but both the calcium and the phosphorus were increased in the McCollum mixture so that the ratio was 6:1.5 (Table V). In spite

of the absolute increase of phosphorus, no change in serum calcium level came about.   A ration was next devised which also maintained the ratio of 4:1, but in which the calcium and phosphorus

TABLE V

Effect of Change in Ratio or in Absolute Amount of Calcium and Phosphorus

Weights of rats	Rickets-producing diet	Rickets shown by x-ray	Diet	Healing shown by x-ray	Serum	
					Ca	P
gm.						
90, 114, 114	McCollum	Moderate	McCollum +	Marked	6.8	10.5
70, 90, 92	(Ca:P =	"	3.3 per cent	"	7.0	8.8
78, 99, 98	4:1)	"	KH$_2$PO$_4$	Moderate	7.3	8.0
66, 80, 80		"	(Ca:P = 4:4 (1:1))	"	7.2	9.3
86, 110, 110	Same	"	McCollum −	Marked	6.6	9.3
64, 80, 70		"	2.36 per cent	"	5.6	9.2
70, 84, 80		"	CaCO$_3$*	"	5.2	9.1
80, 98, 90		"	(Ca:P = 1:1)	"	5.0	9.5
64, 74, 84	Same	"	McCollum +	None	11.6	4.7
60, 72, 76		"	1.4 per cent	Moderate		
62, 90, 90		"	CaCO$_3$, +	Slight		
64, 72, 76		"	0.66 per cent KH$_2$PO$_4$ (Ca:P = 6:1.5 (4:1))	"		
44, 70, 66	Same	"	McCollum†	None	12.3	5.5
44, 70, 64		"	(modified)	Trace		
50, 86, 80		"	(Ca:P = 2:0.5 (4:1))	Slight or moderate	11.3	3.7
64, 80, 84	Same	"	McCollum	None	10.6	4.3
60, 84, 94		Slight or moderate	(Ca:P = 4:1)	"		
58, 80, 90		Slight		Slight or moderate		

* McCollum ration contains 3 per cent CaCO$_3$.
† See text for exact composition.

were both decreased (2:0.5). It contained, therefore, only about one-half as much calcium as the McCollum diet. Its composition was yellow corn-meal 33 per cent, white flour 33 per cent, gelatin 30 per cent, NaCl 1 per cent, $CaCO_3$ 1.3 per cent; to 100 gm. of this ration, 286 mg. of KH_2PO_4 were added. In spite of the fact that this ration contained only one-half as much calcium as the rickets-producing ration, no fall in calcium or nervous symptoms developed (Table V).

Experiments were next carried out to determine whether the fall in calcium and nervous symptoms resulted from an absolute or

TABLE VI

Composite Chart of Absolute Amounts and Ratios of Calcium and Phosphorus in Diets

Diet	Absolute amount		Ratio Ca:P	Tetany
	Ca	P		
⅓ milk,* ⅔ wheat*........................	0.302	0.456	0.67:1	+
⅔ " ⅓ "	0.574	0.566	1:1	+
Milk..	0.960	0.720	1.3:1	+
⅔ milk, ⅓ wheat + 2.9 per cent Ca lactate.	0.960	0.566	1.7:1	±
⅔ " ⅓ " + 4.7 " " " " .	1.184	0.566	2.1:1	—
McCollum.................................	1.232	0.302	4:1	—
" +1.4 per cent $CaCO_3$ + 0.66 per cent KH_2PO_4..........................	1.847	0.453	4:1	—
McCollum (modified)......................	0.610	0.151	4:1	—
" + 3.3 per cent KH_2PO_4.........	1.232	1.232	4:4	+
" − 2.36 per cent $CaCO_3$.........	0.302	0.302	1:1	+

* Dried whole milk and whole ground wheat were used throughout.

from a relative change in calcium or phosphorus. Was the phenomenon due to an increase in the absolute amount of phosphorus or decrease in the absolute amount of calcium? To this end 3.3 gm. of KH_2PO_4 were added to the standard McCollum ration. By this means the phosphorus content was rendered equivalent to that of the calcium in the McCollum diet, and the ratio of Ca:P was maintained at about 4:4; at the same time the absolute amount of calcium remained unchanged. It will be noted from the accompanying table (Table V) that on this ration the calcium in the blood fell just as when the Sherman diets were given. In order to investigate this aspect further, we composed a diet similar to

the rickets-producing ration, but containing far less calcium. The standard McCollum ration is made up by adding 3 per cent $CaCO_3$ to the other ingredients; we incorporated only 0.64 per cent of $CaCO_3$.   In other words it contained 2.36 per cent less $CaCO_3$.   By this means a ratio of Ca:P of 1:1 was brought about and *the ration did not contain any more phosphorus than the rickets-producing McCollum ration (0.302 gm.).   Nevertheless the calcium concentration in the blood fell decidedly* after a period of 2 to 4 days (Table V).   From these various experiments it is evident that the tetany was not the result of changing to a ration in which phosphorus was relatively high compared to calcium nor in which it was greater in absolute amount than in the preliminary diet.   The sudden fall of calcium in the blood with the accompanying nervous symptoms is to be interpreted as the reaction to a sudden fall in the Ca:P ratio in the dietary in comparison to the high ratio of these ions in the rickets-producing diet (Table VI).   This sudden change in ratio, associated with the healing of the rickets, so disturbed the Ca:P balance in the body that the calcium concentration in the blood could no longer be maintained at its physiologic level and hyperirritability of the nervous system ensued.   This phenomenon occurred although the preliminary diet was markedly alkaline and the subsequent diet almost neutral.

This technique, in addition to furnishing a simple and rational method of bringing about tetany as a sequel to rickets, may illustrate a principle which has wider application in dietetics.   It may indicate that a dietary, in spite of being adequate and well balanced, can bring about an abnormal nutritional state owing to the the fact that it does not harmonize with the previous diet of the animal.   Such a phenomenon raises the question of whether it is sufficient to consider adequacy in regard to diets, or whether we must not also take into account the effect of abrupt changes in the dietary.   Possibly, if this point of view is taken into consideration, it may explain some of those puzzling instances, occurring especially in young children, where intolerance is manifested to food which, according to present knowledge, should be well borne.

CONCLUSIONS

Tetany can be induced in rachitic rats simply by an abrupt change from a rickets-producing ration, high in calcium and low in

phosphorus, to a normal ration of dried milk, or of dried milk and whole wheat. The fall in calcium in the serum which is brought about by this means, develops within 48 hours but is maintained for only a few days.

This striking reaction is not due to an absolute nor to a relative increase of phosphorus in the dietary but to a sudden shift in the Ca:P ratio in the subsequent diet as compared to the preliminary diet. When this ratio of Ca:P is decreased from about 4:1 to 1 or 1.5:1 tetany ensues, whereas if it is decreased only to about 2:1 the fall in calcium and nervous symptoms do not come about.

Attention should be directed to the effect of marked alterations in the constitution of dietaries, as such shifts may help to explain nutritional disturbances which are inexplicable merely from the standpoint of adequacy.

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THE DETERMINATION OF THE CALCIUM, MAGNESIUM, AND ACID-SOLUBLE PHOSPHORUS OF MILK BY MEANS OF TRICHLOROACETIC ACID FILTRATES

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INTRODUCTION

The use of a trichloroacetic acid serum filtrate for the quantitative determination of phosphorus in blood, as developed by Bell and Doisy (1) and Benedict and Theis (2), and later introduced by Roe and Kahn (3) for calcium determinations, suggested to the writer the possibility of using a similar procedure in determinations of acid-soluble minerals in cow's milk, in place of the ashing method. The elimination of the ashing method in making such determinations would result in a considerable saving of time and less equipment would be required.

Trichloroacetic acid has been used by Greenwald (4) for the precipitation of blood proteins in order to carry out quantitative determinations of non-protein nitrogenous constituents. Lyman (5) has used trichloroacetic acid as a protein precipitant in both blood and milk for the purpose of preparing filtrates for calcium determinations. Van Slyke and Bosworth (6) have shown that all of the calcium, magnesium, inorganic phosphorus, sodium, potassium, and non-protein nitrogen in milk is found to be in the soluble form in the filtrate when sour milk is filtered.

In the present work the writer has used the trichloroacetic acid filtrate method for calcium, magnesium, and acid-soluble phosphorus determinations in cow's milk, and data are presented to show the accuracy of this method as compared with the ashing method.

Procedure

Trichloroacetic Acid Filtrate Method—20 cc. samples of cow's milk are pipetted into each of two 100 cc. volumetric flasks, the

pipette and flasks having been accurately calibrated. The flasks are filled to the 100 cc. mark with 10 per cent trichloroacetic acid solution, the acid being added slowly and the flask rotated constantly. The contents are thoroughly mixed and allowed to stand stoppered for 30 minutes with frequent stirring. They are then filtered through dry 15 cm. acid-washed filter paper (Schleicher and Schüll No. 589, blue ribbon). The funnel should be covered with a watch-glass during the filtration in order to eliminate, as far as possible, any error due to evaporation.

50 cc. of the filtrate from each sample (corresponding to 10 cc. of milk) are taken for the mineral determination. Then the same procedure is used as in the ashing method after the ash samples have been dissolved and transferred to beakers.

Ashing—10 cc. samples of milk of known specific gravity are pipetted into clean platinum crucibles, dried overnight at 98–100° to remove moisture, and ashed to a white residue. The ash is taken up with dilute hydrochloric acid and transferred to beakers.

Calcium

In certain procedures which have been described for determining blood minerals, notably those of Bell and Doisy (1), Benedict and Theis (2), Roe and Kahn (3), and Greenwald (4), the samples were first diluted with water before the trichloroacetic acid solution was added. In the present work it was felt that by eliminating the addition of water the procedure might be shortened and the degree of accuracy increased. A study was first made of the use of equal parts of milk and acid solution.

Hydrogen Ion Concentration of Milk and Acid Mixtures—A number of samples of milk having pH values averaging 6.54 were mixed with equal quantities of trichloroacetic acid solutions; the normalities of the acid solutions were 0.05, 0.06, 0.10, 0.20, 0.40, and 0.609, respectively. When 0.05 N acid solution was used, the pH values of the resulting mixtures were between 4.75 and 4.95. These mixtures could not be effectively filtered. When 0.06 N acid solution was used, the pH values of the resulting mixtures were 4.31 to 4.62. The latter pH value occurred in a mixture in which a sample of rich Jersey milk was used; when the pH value was below 4.6, the approximate isoelectric point of casein, the material filtered readily but the filtrates were slightly

turbid and calcium oxalate crystals precipitated from such filtrates could not be readily filtered and washed. In these cases, calcium recoveries showed an error of -8.9 to -3.8 per cent.

The most accurate calcium recoveries were obtained with the use of the stronger acid solutions. The 0.609 N (approximately 10 per cent) acid solution, when added to an equal volume of fresh milk, resulted in a mixture the pH of which was approximately 0.80. The filtrate was practically clear, with very little or no turbidity; it was entirely free of fat as determined by the Babcock test.

A number of determinations[1] of the calcium content of normal cow's milk were made by the ashing method and the trichloroacetic acid filtrate method. 1 part of 10 per cent acid solution was added to 1 part of milk. When skim milk was used, the filtration method produced results which were usually slightly high for calcium, not greater than 1.5 per cent; when whole milk was used, the positive error was as great as 4.03 per cent. The results indicated an increased content of calcium in skim milk as compared with the same milk before skimming, this increase corresponding closely with the percentage of fat in the whole milk.

At the time that this work was in progress, a second paper dealing with the determination of blood calcium was published by Roe and Kahn (9). They recommended the use of 4 parts of trichloroacetic acid solution with 1 part of blood serum. The writer made a study of this improved procedure and found that the use of 4 parts of trichloroacetic acid solution with 1 part of milk was more effective in removing the proteins and more accurate for calcium determinations than the use of the 1:1 mixture. Further, in the improved procedure, the calcium oxalate crystals could be digested by heating in order to hasten precipitation, as recommended by McCrudden (8), instead of being allowed to stand overnight. This advantage results from the fact that there is no appreciable amount of heat-coagulable protein present to cause interference in the filtration and washing of calcium oxalate crystals when the 4:1 ratio of acid and milk is used. Typical results are shown in Table I.

[1] Calcium analyses were carried out by the method of Meigs, Blatherwick, and Cary (7), which is a modification of the McCrudden (8) method. Calcium was determined as calcium oxalate, by permanganate titration.

Van Slyke and Bosworth (6) have shown that approximately 33.3 per cent of the calcium and 53.8 per cent of the magnesium in fresh milk occur in a soluble, non-colloidal form. The balance of the calcium, existing principally as colloidal dicalcium phosphate and calcium caseinate (probably Ca₄ caseinate), becomes soluble

TABLE I

Comparison of Filtration Method (1 Part of Milk to 4 Parts of 10 Per Cent Trichloroacetic Acid Solution) with Ashing Method.

Sample	Fat in milk	Ca per 100 cc. milk				
		Ashing method	Average	Filtration method	Average	Variation
	per cent	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
Mixed whole milk*	4.0	129.2		129.6		
		126.8	128.0	130.0	129.8	+1.41
" skim "	0.01	134.8		135.2		
		136.0	135.4	134.0	134.6	−0.59
Holstein whole "	2.95	115.6		116.0		
		115.2	115.4	116.8	116.4	+0.87
" skim "	0.01	118.2		119.0		
		120.8	119.5	119.0	119.0	−0.42
Jersey whole "	4.8	136.0		135.9		
		136.2	136.1	136.1	136.0	−0.07
" skim "	0.01	142.0		141.6		
		141.6	141.8	142.2	141.9	+0.07
Mixed whole "	3.8	125.2		126.4		
		125.8	125.5	126.0	126.2	+0.56

* The same milk before and after skimming.

(as does the balance of the magnesium) in the presence of a sufficient amount of acid. It is believed that the colloidal calcium of calcium caseinate is displaced by H⁺ ions of the acid and appears in the form of soluble monocalcium salts.

Rothwell (10) has presented a method of determining calcium in cow's milk by precipitating it directly as calcium oxalate, and centrifuging. In the case of human milk, calcium recoveries were

incomplete. Lyman (5) has reported low results in the direct precipitation of calcium in cow's milk. In the present work, calcium recoveries in skim milk, by direct precipitation, were within ±2 per cent of those obtained by ashing; in whole milk, however, calcium recoveries by direct precipitation were incomplete, ranging from 91.2 to 96.2 per cent of the ashing values.

Magnesium

Because of the small amount of magnesium occurring in milk, it is advisable, in gravimetric analyses, to use 30 cc. of milk for each analysis instead of 10 cc.

TABLE II

Comparison of Filtration Method with Ashing Method in Determining Magnesium Content of Milk

Sample	Fat in milk	Mg per 100 cc. milk			
		Ashing method	Average	Filtration method	Average
	per cent	mg.	mg.	mg.	mg.
Jersey	5.2	17.0		16.0	
		16.0	16.5	16.5	
				17.0	16.5
Mixed	4.1	14.2		14.4	
		14.4	14.3	14.5	14.45
Holstein	3.0	13.2		13.0	
		13.1	13.15	12.9	12.95

40 cc. samples of milk are pipetted into 200 cc. volumetric flasks and the flasks are filled to the mark with 10 per cent trichloroacetic acid solution. After filtering, 150 cc. aliquot portions, corresponding to 30 cc. of milk, are used. In the ashing method, 30 cc. samples of milk are used. The magnesium determinations were carried out by Method II of the Association of Official Agricultural Chemists ((11) p. 29), except that in the analyses of the trichloroacetic acid filtrates, the first addition of alcohol was omitted.

Consistently accurate results were secured by the new method. Representative data are shown in Table II.

Acid-Soluble Phosphorus

Greenwald (12) has shown that the phosphorus compounds of blood may be divided into three classes: (*a*) lipid, (*b*) an acid-soluble form, probably inorganic in nature, and (*c*) phosphorus combined with protein. There is little doubt that the phosphorus in milk exists in these three forms. Van Slyke and Bosworth (6) analyzed both fresh and sour milk, using a porous porcelain filter to separate the soluble from the insoluble constituents, and found approximately 53.6 per cent of the phosphorus in a soluble form in fresh milk and from 76.8 to 79.7 per cent in the inorganic form. They showed that all the inorganic phosphorus becomes soluble in the process of souring. They found that the ratio of organic to insoluble inorganic phosphorus varied from 1:0.83 to 1:2.47. From their data of analyses of sixteen samples of milk, the amounts of soluble phosphorus plus insoluble inorganic phosphorus are calculated as follows: maximum 83.1 per cent, minimum 73.0 per cent, average 78.3 per cent.

Concerning the organic phosphorus compounds, Bosworth and Van Slyke (13) found that purified casein contains about 0.71 per cent phosphorus; Dornic and Daire (14) reported 0.058 per cent lecithin in milk, and they stated that Stoklasa found 0.09 to 0.113 gm. and that Glikin found 0.05 to 0.15 gm. of lecithin per 100 cc. of milk. Koch and Woods (15) found an average of 0.043 per cent lecithin and 0.036 per cent cephalin, a total of 0.079 per cent of both. Lecithin having a formula $C_{44}H_{90}NPO_9$ (molecular weight 807.99) contains 3.84+ per cent phosphorus. Cephalin contains about 4 per cent phosphorus.

On the assumption that there is approximately 0.07 gm. of lecithin plus cephalin in milk per 100 cc., the phospholipid phosphorus would amount to approximately 2.75 mg. per 100 cc. of milk. A sample of milk containing 3.0 per cent casein would contain approximately 21.3 mg. of casein phosphorus per 100 cc.; a total of 24.05 mg. of organic phosphorus would be present.

Quantitative determinations of acid-soluble phosphorus were made in a number of samples of cow's milk. The samples were prepared as described for calcium, trichloroacetic acid being used as the protein precipitant and mineral solvent. Phosphorus analyses in the filtrates were carried out by the gravimetric pro-

cedure of the Association of Official Agricultural Chemists ((11) p. 3). Representative data are shown in Table III.

TABLE III

Comparison of Filtration Method with Ashing Method in Determination of Phosphorus in Milk

Casein	Fat	P per 100 cc. milk				Per cent total P
		Ashing method	Average	Filtration method	Average	
per cent	per cent	mg.	mg.	mg.	mg.	
2.60	3.65	115.2		92.8		
		115.6	115.4	92.0	92.4	80.1
2.64	3.60	112.7		92.0		
		112.1	112.4	92.0	92.0	81.9
2.00	2.90	95.2		74.4		
		93.0	94.1	75.0		
				75.0		
				73.6	74.5	79.2
2.42	3.10	93.6		72.0		
		90.2	91.9	71.0		
				71.8		
				71.2	71.5	77.8
3.04	4.60	125.9		86.2		
		125.5	125.7	85.8	86.0	68.4
2.55	3.00	92.2		64.3		
		94.2	93.2	65.3	64.8	69.5
2.60	4.00	105.9		78.6		
		108.5	107.2	76.3		
				76.6	77.2	72.0
Average..2.55	3.55		105.7		79.8	75.5

Of the total phosphorus the percentage recovered in the acid filtrates varied between 68.4 and 81.9 per cent; the average was 75.5 per cent. The value 2.55 (average per cent casein) multiplied by 0.0071 equals 0.018 or approximately 17.5 mg. of casein phosphorus per 100 cc. of milk. This figure added to 79.8 mg. (the

average acid-soluble phosphorus) equals 97.3 mg. of acid-soluble plus casein phosphorus. When the above estimated figure for lipid phosphorus is added, the total falls below the figure 105.7 mg. obtained for average total phosphorus.

It is impossible to conclude from the data in Table III whether the figure for acid-soluble phosphorus corresponds exactly with the figure for inorganic phosphorus in milk. Briggs (16) indicated that the acid-soluble phosphorus fraction of blood represents the true value for inorganic phosphorus.

In order to ascertain whether any organic phosphorus was present in the acid-soluble phosphorus fraction, trichloroacetic acid filtrates were prepared in quadruplicate from two different samples of milk. In the case of each, phosphorus was determined in aliquots of two of the filtrates; the other two filtrates were ashed wet in Kjeldahl flasks with sulfuric and nitric acids and the phosphorus was then determined in the ashed samples. The results were identical. There was no appreciable amount of organically combined phosphorus in the trichloroacetic acid filtrates.

A series of trichloroacetic acid filtrates of a sample of milk was prepared in which the milk and acid mixtures were allowed to stand for $\frac{1}{2}$, 24, and 72 hours, respectively, before filtering. The phosphorus contents of these filtrates were all found to be the same. The solution of phosphorus apparently reaches equilibrium within $\frac{1}{2}$ hour.

It was found impractical to determine acid-soluble phosphorus in Folin and Wu (17) filtrates for the reason that in a solution of milk salts the presence of tungsten interferes in the precipitation and separation of phosphorus by means of molybdate mixture. When the trichloroacetic acid filtrate method is used, there are no interfering materials present in the filtrates.

Protein Precipitation—Efficient protein precipitation is an essential factor in preparing milk filtrates for the determination of minerals or of non-protein nitrogen constituents. In the present work it was found that the completeness of protein precipitation is dependent upon the concentration of trichloroacetic acid in the milk and acid mixtures. Below pH 4.6, protein solubility gradually decreased with decrease in the pH of the mixture. This is contrary to the effect observed when acids such as hydrochloric are used. Protein precipitation was most effective in the case of

milk having a low buffer value; the precipitation was further favored by increasing the dilution of the system. For all samples of cow's milk, the use of 4 parts of 10 per cent trichloroacetic acid with 1 part of milk produced practically complete protein precipitation.

Trichloroacetic acid solution added to milk produces a much coarser precipitate than does the mixture of sodium tungstate and sulfuric acid used in the Folin and Wu (17) method; the trichloroacetic acid mixture filters much more rapidly and yields a considerably larger amount of filtrate in proportion to the amount of milk used.

SUMMARY

A procedure is described for determining calcium, magnesium, and acid-soluble phosphorus in milk without ashing. The procedure is much more rapid than the ashing method. A saving of at least 1 working day in preparing the samples is effected. The use of 4 parts of 10 per cent trichloroacetic acid solution with 1 part of milk was found most effective in preparing protein-free filtrates for mineral determinations. A high degree of accuracy was attained in determinations of calcium and magnesium.

In a number of milk samples, 68.4 to 81.9 per cent of the phosphorus was found to be acid-soluble. There was no definite correlation between the amount of acid-insoluble phosphorus and the amount of casein; in general, however, milk high in casein was high in acid-insoluble phosphorus. The procedure outlined herein should be of value in studying phosphorus combinations in milk, with particular reference to casein and lipid phosphorus.

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ON THE OXIDATION BY POTASSIUM FERRICYANIDE OF CERTAIN CONSTITUENTS OF THE SERUM IN ANEMIA

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In attempting to make measurements of the oxygen content of the blood of anemic rabbits by the method devised by Haldane (1898, 1920), it was found that after the liberation of the oxygen by the addition of potassium ferricyanide no steady end reading could be reached. The final solution began to absorb gas from the flask in quantities which, in a short time, might exceed the total volume of oxygen previously liberated. Such a difficulty has been described by Douglas (1910) also after studying the blood of anemic rabbits. More recently the reactions involved have been investigated by Parsons and Parsons (1927) and by Litarczek (1928).

Litarczek found that this reaction also occurred in the course of determinations made on normal blood though much less vigorously than in those made on anemic blood. He found, moreover, that it could be considerably intensified if the blood examined were taken from a normal individual shortly after a meal during which considerable quantities of fat had been consumed. Since the extraction of lipoids from the serum by ether greatly reduced the intensity of the reaction, Litarczek concluded that it depended upon the oxidation of fatty acids present in the blood, though he was unable to demonstrate that the lipoids so removed later exhibited any marked oxidative reaction in the presence of potassium ferricyanide.

Parsons and Parsons reached similar general conclusions, though they were able to show that, if the ether extraction of the serum

were carried out by a method which excluded contact with air, the subsequent oxidation of the lipoid emulsion proceeded much more actively.   They further undertook fractionation of the serum by the addition of varying concentrations of ammonium sulfate. After full saturation of the serum the filtrate was entirely inactive, while that obtained after one-third saturation exhibited almost as much activity as the original serum.   In the former case the precipitate contained all the substances oxidizable under these conditions, and they interpret these results as being consistent with their view that the lipoids are the substances present in the serum which are responsible for the reaction.

Since the Haldane blood gas apparatus is widely used it is desirable to investigate any limitations in its applicability to the determination of blood gases.   Such a study may result in a further insight into the changes in the blood after extensive bleeding, and so lay the basis for some modification of the original method which would render it more suitable for investigating the blood in anemia. This work was carried out with both these points in view, yet, though the reason for the systematic error now appears clearer, we were not able to develop any satisfactory modification of the method.

Methods

All estimations of the oxygen consumption of the blood, serum, or other solutions used, were made upon the respiration apparatus described by Warburg (1926).   The quantities of the solutions used have been the same as those required for the Haldane blood gas apparatus (1920): 2 cc. of blood, serum, etc., 0.75 cc. of sodium carbonate (1 per cent)solution, and 0.25 cc. of a freshly prepared saturated solution of potassium ferricyanide.   The potassium ferricyanide was placed in a side arm of the respiration flask and added to the solution in the main chamber after temperature equilibration at 37° had taken place.

EXPERIMENTAL

The participation of the red blood cells in this reaction, which might readily have been inferred from the observations of Morawitz (1909) upon the greatly heightened respiration of regenerating blood, has been excluded by Parsons and Parsons, and by Litarczek. The reaction is one which concerns some component of the serum

as can be seen from the illustrative experiment given in Table I, in which a comparison was made of the oxidation of whole blood, serum, and red cells from the same blood but resuspended in normal physiological salt solution.

TABLE I

Oxidation of Whole Blood, Serum, and Cells from Same Blood Resuspended in Normal Physiological Salt Solution

Time	C.mm. oxygen consumed per cc.		
	Whole blood	Serum	Cell suspension
min.			
15	24.5	83.5	.6.7
30	40.0	135.4	12.5
45	56.1	167.8	16.3
60	72.8	192.6	19.8
75	89.4	211.7	21.8

The red cell count was 2.21 millions per c.mm. The reticulocyte count was 38 per cent. The temperature of the water bath was 37.5°.

It is evident from this experiment that the red blood cells are not greatly concerned in the reaction, indeed probably a large part of the oxygen consumption in the flask containing the cell suspension took place in the small quantity of serum still adherent to the cells. That less oxygen was consumed by the whole blood than by the serum alone was probably due to the smaller quantities of serum present and also to the lower effective concentration of potassium ferricyanide, part of which had reacted with the hemoglobin present. Both of these factors also probably contribute to the intensification of the reaction when anemic blood rather than normal blood is examined.

Constituent of Serum Responsible for Reaction

The serum in anemia exhibits two important changes: a relative lipemia, and a considerable increase in the purine nitrogen. The former was first described by Boggs and Morris (1909) in the blood of rabbits rendered anemic by bleeding, and has since been somewhat extensively studied, notably by Bloor (1921, 1925). An increase in the concentration of the serum purine bodies has been described by Krafka (1929) in a dog recovering from an experi-

mental anemia, and by Riddle (1930) in patients recovering from pernicious anemia as a result of liver treatment.

In order to determine whether the change in the purine concentration might play a part in the enhanced oxidation, the serum from an anemic rabbit was dialyzed inside a collodion sac against an equal volume of normal physiological sodium chloride solution. Since the purine bodies are small readily diffusible substances it was expected that, were they the substances concerned, the oxidation reaction of the solutions on the two sides of the membrane would rapidly attain the same value. The following tabular matter gives the results obtained.

Duration of dialysis	C.mm. oxygen consumed per cc.			Sugar concentrations	
	Original serum	Inside	Outside	Inside	Outside
hrs.				per cent	per cent
18	202.0	178.0	32.4	0.097	0.054
72		139.2	75.2	0.079	0.083

The sugar determinations were made by the method of Hagedorn and Jensen. The duration of each experimental determination was 75 minutes.

This experiment shows that the rate of diffusion of the oxidizable material, even through a fairly permeable collodion sac, is slow, and that when equilibrium has been attained for the serum sugar the activity of the serum inside the membrane is still nearly twice that of the solution outside. Further evidence against the participation of the purine bodies was the absence of any absorption of oxygen by the urine of an anemic animal upon the addition of potassium ferricyanide.

Oxidation of Serum in Relation to Lipoids

Fishberg and Fishberg (1928) have studied the lipemia which occurs in rabbits after repeated bleeding. They found that the total fat content of the blood may increase 8 or 9 times, figures which are in good agreement with those of Boggs and Morris. Since it is well known that the unsaturated fatty acids are more readily oxidizable *in vitro* than saturated ones, it seemed possible that they might be the constituents involved in this reaction. Were this so the oxidizability of the serum should bear some relation to the degree of iodine absorption by the fats which it con-

tains.   This possibility was explored by comparing the oxygen consumption of the serum when potassium ferricyanide was added to it with the iodine absorption of the fats extracted by the method devised by Bloor, Pelkan, and Allen (1922).   The oxygen consumption of the serum was determined as before by the Barcroft-Warburg apparatus, and the iodine absorption by the method of Gibson and Howard (1923) upon the extracted serum fats.   Several rabbits were used, both in their normal condition and after the production of a severe grade of anemia by successive bleedings. The results will be seen in Table II.

TABLE II
Oxidation of Serum in Relation to Iodine Absorption of Serum Lipoids

Rabbit No. (a)	Red cells (b)	O₂ consumed per 100 cc. serum* (c)	I₂ absorbed per 100 cc. serum (d)	Ratio $\frac{(c)}{(d)}$
	millions per c.mm.	c.mm.	mg.	
21	4.98	14,200	133	107
15		23,300	178	131
15	2.81	24,400	187	131
34		27,900	271	103
21	3.57	34,700	253	138
34	2.68	34,900	241	145
34		37,700	308	122
21	2.11	38,700	360	108
21		43,600	345	126
21		44,400	323	137
23	2.73	44,500	310	143
23		45,200	290	156
21		46,200	350	132

* The duration of the oxygen consumption determinations was 4 hours.

It can be seen from Table II that the oxygen consumption of an anemic rabbit's serum after the addition of an alkaline solution of potassium ferricyanide, is considerably greater than that of a normal fasted animal, and that coincident with this rise is an approximately similar rise in the iodine absorption value of the serum fats.   The ratio between the two is to be found in the last column.

Should the unsaturated fatty acids be the constituents of the

serum which are oxidized it might be expected that after oxidation they would no longer be able to absorb as much oxygen as before. This was tested experimentally. At the same time as the oxygen consumption of the specimen of serum from an anemic rabbit was measured in the Barcroft-Warburg apparatus, 7.5 cc. of a mixture of serum, sodium carbonate, and potassium ferricyanide, in the same proportions as in the respiration flask were shaken in a larger flask at the same rate in the same water bath. The only difference between the two was that one flask was larger and contained more than twice as much of the solution as the other. At the end of 4 hours the oxygen consumption of the serum was determined, and the iodine absorption of the fatty acids remaining in the mixture in the larger flask was estimated. This iodine absorption was compared with that of the fatty acids present in the original serum. The results will be found in Table III.

TABLE III

Influence of Oxidation of Serum on Iodine Absorption of Serum Lipoids

Oxygen consumed per 100 cc. serum (4 hrs.)		Iodine absorption by serum fats			Ratio $\frac{(b)}{(e)}$
		Before	After	Difference	
		Mg. iodine per 100 cc. serum			
(a)	(b)	(c)	(d)	(e)	
cc.	*mg.*				
34.7	49.6	253	151	102	0.486
46.2	66.1	350	184	166	0.398
44.4	63.5	323	155	168	0.378

It is clear from these determinations that after oxidation of the serum mixture has taken place, the capacity of the serum fats for taking up iodine is definitely reduced. Further it can easily be calculated that about 10 atoms of oxygen have been taken up for every 3 atoms of iodine which might have been absorbed. It is evident therefore that the oxidation has not resulted entirely in the production of the corresponding hydroxy acids, as Lewkowitsch (1909) has described as the principal product of the oxidation of oleic acid by potassium dichromate. The oxidation of the serum fats must have progressed considerably further.

Form of Oxygen Consumption Curve for Serum

The consumption of oxygen by the serum takes place very rapidly at first, but the rate decreases progressively.   This is well shown in Chart I, which indicates the oxygen consumption, both for normal and anemic serum for a period of 20 hours.   The decrement in the rate does not follow any simple logarithmic rule, a result which might be expected from the probability that a number of unsatu-
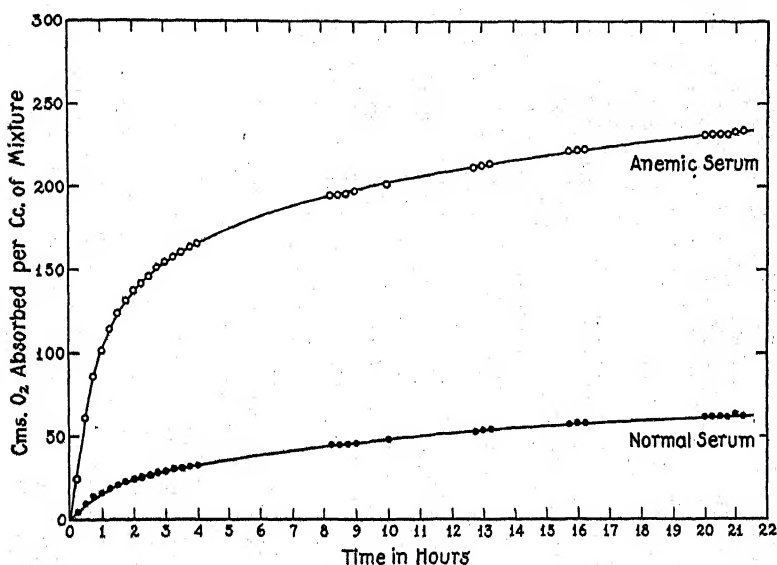
CHART I. Curves showing the oxygen consumption for normal serum (●——— ●) and anemic serum (o——— o) after the addition of sodium carbonate and potassium ferricyanide.

rated fatty acids are simultaneously oxidized, some of them more readily than others.

The reaction still continued at a reduced intensity after 20 hours so that complete oxidation was not attained, nor is it possible to extrapolate from the data to discover when this point would be reached.   The curve probably represents a summation of the different oxidation curves for fatty acids ranging from oleic acid on the one hand, to those with several unsaturated linkages, such as arachidic acid described by Hartley (1909) on the other. Further the rate of oxidation may be influenced by the occurrence

of autocatalytic reactions such as will be described below in the oxidation of oleic acid.

Attempts to Inhibit the Serum Oxidation Reaction

Several efforts were made to inhibit, or at least reduce, the intensity of the reaction. All were unsuccessful. The addition of potassium cyanide to a final concentration of 0.005 M, and the additon of phenylurethane up to saturation in the sodium carbonate used, both of which Warburg had found to be inhibitors of both biological oxidation systems and his charcoal model, were without effect upon this reaction. The reason may have been that these substances are only effective in inhibiting oxidation reactions catalyzed by colloidal particles upon which they can be adsorbed. On the other hand, as Kuhn and Meyer (1929) have suggested, it is possible that potassium cyanide is without effect upon the oxidation of fats, inhibiting only that of amino acids and carbohydrates.

Oxidation of Oleic Acid by Potassium Ferricyanide

Since oleic acid is one of the principal unsaturated fatty acids in the serum, experiments were carried out to examine its oxidizability by potassium ferricyanide under conditions similar to those used for the serum. Two specimens of oleic acid were used, one with an iodine number of 66, and specific gravity of 0.892 at 20°, and another with one of 88 and specific gravity of 0.894 (the theoretical iodine number is 91). The oleic acid was dissolved in 1 per cent sodium carbonate solution in concentrations of about 0.4 gm. per cent. 2 cc. of this solution, together with 0.75 cc. more of 1 per cent sodium carbonate solution, were placed in the flask of a Barcroft-Warburg apparatus and after temperature equilibrium at 37° had been reached, 0.25 cc. of a saturated solution of potassium ferricyanide at the same temperature was added. The oxygen consumption of the final mixture was measured. A control measurement was made in which the potassium ferricyanide was omitted. Table IV gives the oxygen consumptions in the experimental and control flasks.

Chart II shows the course of the reaction. From this it can be seen that the reaction does not proceed uniformly, but when charted shows a sigmoid curve. Such variations in the speed of progress

of the oxidation might be due to the products of the reaction affecting its further course.   In order to obtain more definite evidence

Experiment No.	C.mm. oxygen consumed per cc. oleate solution	
	With $K_3Fe(CN)_6$	Without $K_3Fe(CN)_6$
1	156.5	−2.5
2	180.5	Nil
3	114.0	1.2
4	179.2	0.7

The experiment lasted 210 minutes.
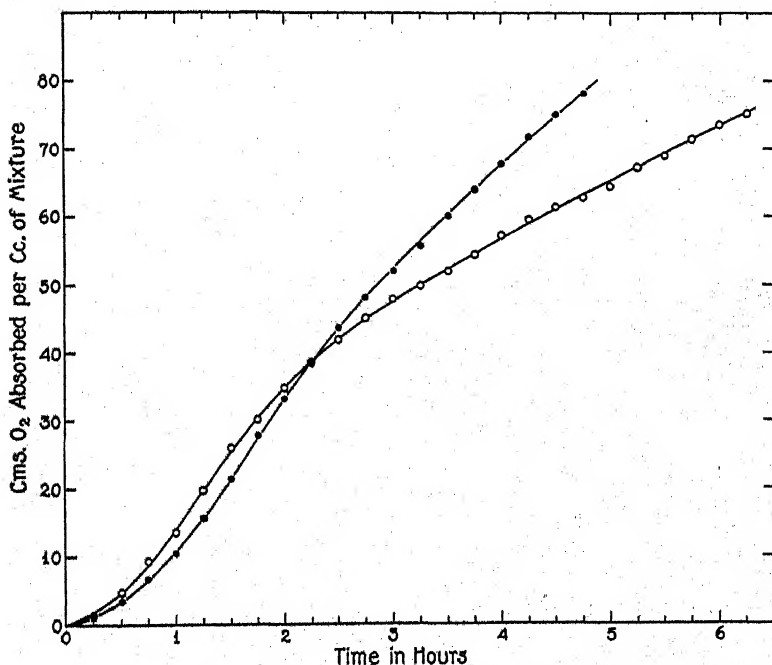
CHART II. Curves showing the oxidation of oleic acid in the presence of sodium carbonate and potassium ferricyanide.

dence upon this point, a further experiment was performed in which in place of the 0.75 cc. of sodium carbonate usually added,

0.75 cc. from the experimental and control flasks of the previous experiment were added to fresh oleic acid and the oxidation of the two solutions again measured after the addition of potassium ferricyanide.   In this way the fresh oleic acid solution was seeded with any possible autocatalytic products developed in the previous reaction.   The results of this experiment are given in Chart III.

It can be seen from Chart III that the course of the reaction in the two flasks is quite different.   In the flask seeded from the
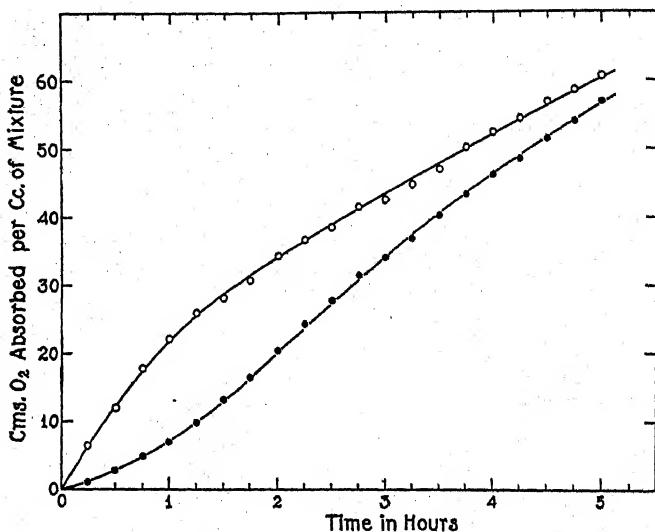
CHART III.   Curves showing the influence of products of reaction upon oxidation of oleic acid by sodium carbonate and potassium ferricyanide. Oxidation curves (1) oleic acid mixture seeded from a similar mixture in which oxidation had previously occurred (o———— o); (2) oleic acid mixture seeded from a mixture in which no oxidation had previously taken place (•———— •).

original flask, in which no oxidation had previously taken place, the form of the curve reduplicates the original sigmoid, while in the other flask to which products of oxidation from the previous experiment had been added the curve is of paraboloid appearance. It would seem that while in the former it took time for the formation of products capable of affecting the subsequent course of the reaction, in the latter they were added, already formed, in the seeded solution.

The total quantity of oxygen taken up by the oleic acid during the period of observation is very much less than that taken up by the serum fats having the same capacity for the absorption of iodine. Only 1 atom of oxygen is used for every 2 or 3 potential iodine atoms, which is roughly the reverse of that for the serum fats. Consequently the oleic acid present in the serum is probably in only a minor measure responsible for the oxidation reaction with potassium ferricyanide, and other more unsaturated fatty acids account for the greater part of it.

<div align="center">DISCUSSION</div>

The serum lipoids both of normal and anemic dogs have been extensively studied by Bloor (1923). In both he found that large proportions of the fatty acids present were unsaturated and probably largely oleic and linoleic acids with small quantities of even more unsaturated ones such as linolenic acid. In the serum in anemia however the proportion of linoleic to oleic acid was considerably increased, and at the same time the quantity of fatty acids in the serum was also somewhat raised. The rise he observed was, however, not very great, possibly because the dogs he examined were in a condition of chronic anemia in which, following Bloor's suggestion it is highly probable that extension of the erythropoietic tissue to the available marrow space had already taken place, leaving practically no further marrow fat to be removed by the blood. Horiuchi (1920) in examining the blood of rabbits in anemia found that daily hemorrhages induced a lipemia which attained a maximum in the course of a few days and then subsided to about its original value, even though bleeding was still kept up. In this case the lipemia appeared coincidently with the extension of the bone marrow, falling away after this had taken place and little further fat remained to be replaced. Unfortunately Horiuchi makes no mention of the degree of unsaturation of the lipoids at the height of the lipemia, though Boggs and Morris state that the iodine absorption of the serum fats is definitely elevated at this time.

The rise in the oxidizability of the serum in the presence of ferricyanide appears concurrently with the increase in concentration of the unsaturated fatty acids. That potassium ferricyanide is able to oxidize unsaturated fatty acids is definitely

shown by its oxidation of oleic acid dissolved in sodium carbonate solution. That it would also be able to oxidize linoleic and lino- lenic acids and any more unsaturated ones seems very probable. Kuhn and Meyer (1929) were able to catalyze the oxidation of oleic, linoleic, and linolenic acids with hemin; the more unsaturated the fatty acid, the more rapid was the subsequent oxidation. War- burg (1914) was able to catalyze the oxidation of linolenic acid by iron (ferrous ammonium sulfate) in acid solution, though oleic acid remained unoxidized under such conditions. Just as for the serum fats described in this paper, Warburg found that the oxida- tion of the linolenic acid was accompanied by a decrease in the iodine absorption values of the solution. A similar decrease was described by Meyerhof (1923) for the oxidation of linolenic acid by cysteine and by thioglycolic acid.

It seems therefore that potassium ferricyanide has an oxidation potential sufficient not only to convert the reduced hemoglobin to methemoglobin (Conant, 1923), but also to induce oxidations of other serum constituents which involve the taking up of free oxygen. Where these substances are present in any quantity, as in anemia, the Haldane method for blood oxygen determinations will be unsatisfactory and recourse must be made to Van Slyke's modification in which the ferricyanide-serum mixtures present a much smaller surface of contact with the oxygen which in turn is at a low partial pressure. In this way the opportunity for sub- sequent oxidation is greatly minimized.

SUMMARY

1. The constituents of the serum of rabbits made anemic by hemorrhage which interfere with the use of the Haldane blood gas apparatus for blood oxygen determinations appear to be un- saturated fatty acids.

2. The progress of the anemia is accompanied by an increase in the iodine absorption value of the serum fats and by an almost quantitatively similar increase in the oxidizability of the serum by potassium ferricyanide.

3. After oxidation of the serum by potassium ferricyanide the iodine absorption values of the serum fats fall.

4. The oxidation of an unsaturated fatty acid, oleic acid, under similar conditions is described and its relation to the oxidation

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THE EFFECT OF HEAT UPON THE BIOLOGICAL VALUE OF CEREAL PROTEINS AND CASEIN

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A number of studies on the heat denaturation of proteins have been reported in recent years, dealing chiefly with the conditions affecting denaturation and flocculation and the probable nature and extent of the molecular changes involved in the phenomenon. Very few attempts have been made to determine the differences in specific properties of the native and denatured proteins or to observe any changes which may have occurred in the biological value of the proteins in animal nutrition. Anson and Mirsky (1) point out that judged by response to the two most sensitive tests for the specificity of proteins only native proteins are highly specific. One of the tests referred to is observation of the position of absorption bands of oxyhemoglobin and carboxyhemoglobin which vary for individuals and species in the native proteins but which are found always in the same position after denaturation. The other test is that of precipitin formation. Injection of egg albumin of different species for example give specific precipitins but injection of denatured egg albumin exhibits only a group specificity. Upon reversal of denaturation the specificity reappears.

Anson and Mirsky (1) conclude from their work upon denatured hemoglobin that denaturation is a process of depolymerization involving the uncovering of a non-polar group in the denatured protein with resulting change in colloidal properties from the lyophyllic to the lyophobic group. In a later communication (2) they have demonstrated the reversal of the denaturation of hemoglobin by the preparation of crystalline hemoglobin, with characteristic native protein properties, from the coagulated form. Although they do not discuss this phase of the subject this would involve the assumption of repolymerization of the native protein from the denatured state.

Other contributors on this subject have assumed denaturation to be in the nature of a mild hydrolysis. Chick and Martin (3) have shown that water must be present when denaturation occurs and Wu and Wu (4) have added evidence that some splitting of the molecule occurs with increase in

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the chromogenic value of the protein. Part at least of this increase in color production resulting from the action of the phenol reagent upon the denatured protein solution was shown to result from material separated from the protein, probably tyrosine and tryptophane. They suggest that other amino acids not taking part in this reaction may also have been separated at the same time. Lepeschkin (5) likewise favors the view of denaturation as a mild hydrolysis. Lewis (6) suggests that denaturation involves hydrolysis of linkages of a character analogous to those involved in the hydrolysis of polysaccharides and not the rupture of polypeptide linkages. He found for example that native and denatured hemoglobin have the same number of free acid and basic groups per molar equivalent.

In all the heat denaturation experiments referred to the temperatures used were relatively low, usually 70° to 80°. However, Chick and Martin (3) made several tests of the effect of heating to 110°, 120°, and 130° crystallized hemoglobin and egg albumin in the absence of added water in order to determine the rôle of water in denaturation. They report that dry heating does not render these proteins insoluble, egg albumin for example heated to 120° for 5 hours remaining 78 per cent water-soluble. Dry hot air currents were passed through the crystallized albumin which still contained 20 per cent water. They concluded that denaturation is essentially a hydrolytic phenomenon. Cohnheim (7) quotes an experiment of Michel and Wichmann in which crystalline egg albumin was heated dry to 150° without change in the character of the protein.

Chick and Martin (3) and Hartridge (8) examined the temperature coefficient of the denaturation process and reported it to be very high, 1.91 per degree centigrade for egg albumin and 1.3 for hemoglobin. This is a rate of over 600 for 10°, the largest temperature coefficient so far recorded. Obviously the range of temperatures within which such observations can be made is quite limited, since denaturation is complete at about 80°.

The nature of the changes which occur in relatively dry proteins at elevated temperatures is apparently unknown. The effect of these changes upon the biological value of the proteins has been considered briefly by Chick (9) and by Goldblatt and Moritz (10). In the former investigation casein heated at 120° to 130° for 36 to 72 hours was fed to young rats in diets deficient in vitamins A and D and compared with similarly used unheated casein. No differences were noted. Goldblatt and Moritz (10) used casein heated at 110° and at 130° for 36 hours in an adequate diet with similar results. The protein level is reported as 20 per cent of technical casein (Merck), corresponding probably to about 2.5 per cent nitrogen or 16 per cent protein. This level is too high for the observation of small differences in nutritive value of the proteins used, as is also that reported by Chick. The temperatures chosen are lower than that used in the experiments here described, and are those commonly used in the oxidative destruction of vitamin traces in casein included in the basal diets fed rats used for vitamin A testing.

In this connection the findings of Coward, Key, Morgan, and Cambden (11) on the apparent vitamin content of various samples of casein are of

interest. The "light white" casein upon which their rats resumed growth showed decreased value after having been heated at 105° for 7 days. The "glaxo" casein which did not allow normal resumption of growth on adequate vitamin A intake may have been heated at higher temperatures, but no description is given of the vitamin-freeing processes to which it had been subjected. However since extraction methods removed only a trace of the growth-promoting fraction of the light white casein it is possible to question whether the discrepancy observed may not have been due to a change in the value of the protein itself. This suspicion is supported by the fact that a rather low level of casein was used, 15 per cent, corresponding to about 12 per cent true protein, a figure near the maintenance level.

It might be thought that any changes in nutritive value produced by heat treatment are ascribable to decreases in digestibility of the heated proteins. But in the cases of egg white (12), and of phaseolin, the most important globulin of the navy bean (13), and the proteins of the velvet bean (14) and the Lima bean (15) the digestibility is much improved by cooking. In fact there is no record of experiments known to the writer which indicate a decrease in the digestibility of protein due to heat treatment either moist or dry. As will be shown later the discrepancy in nutritive value of the heat-treated proteins described in this report is not to be ascribed except in small part to losses in digestion.

Preliminary Growth Experiments on Cereals—The first experiments on this subject carried on in this laboratory were made upon certain prepared breakfast cereals and first brought out the difference in growth-promoting value of the raw, water-cooked, and toasted cereal proteins (16). As may be seen in Table I wheat, rice, and corn were tested alone and supplemented by small amounts of casein. In all cases the diet was made up as follows:

per cent

Cereal.. 95
Agar... 2
Salt mixture (Osborne and Mendel (17))..................... 3

In addition there were fed separately daily to each rat 0.5 gm. of dry brewery yeast as source of water-soluble vitamins and 25 mg. of cod liver oil as source of fat-soluble vitamins. In the casein-containing diets, 5 per cent of dry commercial casein, not heat-treated, was substituted for 5 per cent of the cereal. The feeding period was 8 weeks and the rats used were from 21 to 28 days old and weighed 35 to 60 gm. when placed on the diet. Careful food

intake records were kept and Kjeldahl determinations for total nitrogen made upon each diet. The comparison of value of the

TABLE I

Preliminary Growth Experiments upon Raw and Toasted Cereals

Diet	Nitro-gen	No. of rats	Average gain per day in 56 days	Average daily food intake		Average gain per gm. protein
					Pro-tein (N × 5.7)	
	per cent		*gm.*	*gm.*	*gm.*	*gm.*
Toasted white bread.	1.70	6	0.87	6.9	0.85	1.02
" bread and casein.	2.16	4	3.00	12.7	1.76	1.70
White bread	1.20	8	1.35	10.3	0.90	1.50
" " and casein.	1.77	4	2.64	11.8	1.38	1.91
Puffed wheat	1.70	10	0.50	5.4	0.72	0.69
" " and casein.	2.70	4	2.09	8.5	1.26	1.66
Bread crust.	1.93	9	0.68	4.3	0.67	1.01
" " and casein.	2.33	3	1.70	6.3	1.03	1.65
" crumb.	1.87	7	1.37	6.6	0.90	1.52
" " and casein.	2.35	3	1.80	6.1	1.01	1.78
Raw cracked wheat.	1.60	10	1.87	10.1	1.11	1.68
" " " and casein.	2.04	8	2.86	11.6	1.55	1.84
Toasted cracked wheat.	1.81	7	1.23	8.7	1.09	1.12
" " " and casein.	2.30	7	2.73	11.2	1.62	1.69
Water-cooked wheat.	1.76	6	2.72	13.2	1.52	1.78
Water-extracted toasted wheat.	1.57	4	0.78	7.4	0.86	0.90
" " " and casein.	1.90	4	2.07	9.7	1.18	1.76
Raw wheat and extract of toasted wheat.	1.60	4	1.44	8.8	1.00	1.44
Raw wheat and extract of toasted wheat and casein.	2.00	4	2.73	10.7	1.42	1.92
Raw rice.	1.30	2	1.57	12.4	1.11	1.41
" " and casein.	1.65	4	2.82	13.5	1.47	1.92
Puffed rice.	1.34	2	0.39	6.8	0.71	0.55
" " and casein.	1.80	4	1.39	10.2	1.24	1.12
Raw yellow corn.	1.86	4	1.94	13.4	1.62	1.20
" " " and casein.	2.42	4	2.43	12.7	1.95	1.24
Toasted corn flakes.	1.46	8	0.55	5.7	0.67	0.82
" " " and casein.	2.00	4	1.66	9.7	1.30	1.28

proteins for growth was made by the numerical method proposed by Osborne, Mendel, and Ferry (18) and used extensively by Hoag-

land and Snider (19) and others. The average gain in weight of growing rats per gm. of protein eaten during the 8 weeks period is taken as an index of the biological usefulness of the food protein used in an otherwise adequate diet.

The actual weight gains made vary with the level of protein used as well as with its nutritive quality but the former factor is offset usually by the lower intake figure. Thus the rats fed white bread (Table I) gained 1.35 gm. per day, ate 0.90 gm. of protein and thus gained 1.50 gm. per gm. of protein eaten in 8 weeks. The rats fed white bread and casein gained 2.64 gm. per day but ate 1.38 gm. of protein and so made a gain of 1.91 gm. per gm. of protein eaten.

White bread was compared with the same toasted in $\frac{1}{4}$ inch slices at 150° and bread crust similarly compared with the corresponding crumb. In each case the toasted product caused less total growth and less growth per gm. of protein eaten. When 5 per cent raw casein was introduced into each of these four diets the discrepancy caused by the heat treatment largely disappeared. A moderate amount of supplementing effect by the casein was observed in the untoasted wheat products.

Puffed wheat, said to be prepared by expansion and toasting by steam under pressure, was also found to produce but little growth, 0.69 gm. per gm. of protein eaten as compared with whole raw wheat, 1.68 gm. Whole cracked wheat was then toasted at 200° for 45 minutes in thin layers with stirring at 10 minute intervals. Enough wheat was toasted at one time to provide food for the group of rats fed thereon for 56 days. The cracked wheat was fed raw, toasted, and water-cooked as 95 per cent of the diet previously described. The water cooking was carried out in a large double boiler for 30 minutes, plenty of water being added and the resulting product dried in thin layers before a fan. After being dried it was ground and used in the diet as were the raw and toasted wheat. The temperature reached during the water cooking was close to 90°. This would seem to be sufficient to produce the usual heat denaturation. However the growth value, 1.78, of the water-cooked wheat was even better than that of the raw, 1.68 (a somewhat high figure), and both were greater than that of the toasted wheat, 1.12. The addition of 5 per cent raw casein improved the performance of the toasted wheat materially but added only a little

to that of the raw wheat. The slightly better value shown by the cooked product may possibly be ascribed to improved digestibility.

Since the suspicion arose that toxic products might be present in the caramel-like substances produced by toasting, a few rats were fed toasted wheat which had been water-extracted until all soluble brown extract was removed. These animals grew even less rapidly than those fed the unextracted toasted wheat. Other rats were fed the raw wheat diet plus the caramel-containing extract from a like amount of toasted wheat but with only a little change in their resulting growth, 1.44, as compared with 1.68, for the raw wheat and 0.90 for the extracted toasted wheat. The latter diet evidently lost some valuable material during the water extraction. Again the addition of 5 per cent casein improved largely the extracted toasted wheat diet and less so the raw wheat plus extract diet.

Raw cracked rice was found to provide excellent growth, improved by the addition of casein. Puffed rice, however, gave far less growth which was only partly restored to normal by the addition of the casein.

Raw ground yellow corn, only a little improved by the casein, provided less growth per gm. of protein consumed than did the wheat, a result to be expected from the similar tests of Osborne and Mendel (20), Maynard, Fronda, and Chen (21), and the generally recognized superiority of wheat over corn proteins as shown by the maintenance type of experiment recorded by Thomas (22) and Mitchell (23). Two examples of toasted corn flakes gave less growth than did the raw corn, 0.82 as compared with 1.20.

Effect of Intake—It is obvious that the food intake of all toasted cereals was considerably less than that of the corresponding untoasted products, the former being usually 60 to 80 per cent of the latter. This naturally decreased the absolute amount of growth obtained upon the toasted diets but does not explain the discrepancy in the growth per gm. of protein consumed. Comparison of the latter factors in cases such as those of bread crumb and toasted bread in which the intake happen to be nearly the same, show that the growth obtained is still considerably less in the toasted than in the untoasted products, 1.02 as compared with 1.52.

Wheat Gluten Growth Experiments—A new type of diet was next

used in which was included commercial wheat gluten or a mixture of separately prepared purified wheat proteins, raw or toasted. These diets were made up as follows:

	per cent
Protein	9–24
Agar	4
Salt mixture	4
Crisco	20
Wheat starch	48–63

0.5 gm. of yeast and 25 mg. of cod liver oil were given separately to each rat daily.

In the earlier tests corn-starch, raw or dextrinized, and dextrinized wheat starch were used instead of the raw wheat starch but in most of the later experiments only the latter carbohydrate was included. Gliadin, glutenin, globulin, and leucosin were prepared separately from wheat flour according to the Osborne (24) method and were then combined in the proportions reported by Osborne as characteristic of the spring and winter wheat kernel. Later in the investigation commercial wheat gluten was substituted for the mixture of isolated wheat proteins, with no observable changes in the growth-promoting value of the diets.

Since the rate of growth of young animals is controlled not only by the quality of the protein fed but also by the amount of food ingested and the level of protein in the diet, a complete picture of the effect of heat upon the gluten could be obtained only by the use of a number of protein levels. Accordingly 9, 12, 15, 18, 21, and 24 per cent (crude) of raw and toasted gluten were incorporated in the diets fed groups of young rats. The toasted gluten was heated in a thin layer in an electric oven for 30 minutes at 150° with occasional stirring. The resulting product was a light brown in color. Each diet was made up in lots sufficient for the entire 56 days feeding period and total nitrogen determined on it.

As is shown in Table II the food intake was quite uniform on all these levels and the depressing effect of the toasting upon appetite was quite small as compared with the differences observed in the whole cereal diets toasted at a higher temperature for a longer time. The total protein intake increased with increase in level of protein used and the average daily gain in weight likewise increased to a maximum at the 18 to 21 per cent level. But the

TABLE II

Growth of Young Rats on Diets Containing Various Levels of Raw and Toasted Wheat Gluten

Condition of gluten	Protein level		No. of rats	Average food intake per day		Average body weight			Average gain per day	Gain per gm. protein	Difference between gains on raw and toasted gluten
	Crude	Actual (N × 5.7)		Total	Protein	Initial	End	Gain in 56 days			
	per cent	per cent		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Raw.........	7-9	5.6-6.7	15	6.2	0.56	59	100	41	0.73	1.31 ±0.03	0.37 ±0.04
Toasted.....	7-9	5.4-6.7	17	6.2	0.57	59	89	30	0.53	0.94 ±0.03	
Raw.........	10-12	8.5-10.2	25	6.0	0.75	50	104	54	0.96	1.29 ±0.03	0.25 ±0.05
Toasted.....	10-12	8.5-10.2	20	6.5	0.81	58	106	48	0.85	1.04 ±0.04	
Raw.........	15	11.1-12.4	12	7.4	1.02	58	132	74	1.32	1.29 ±0.03	0.35 ±0.03
Toasted.....	15	11.1-12.4	12	6.2	0.90	50	98	48	0.85	0.94 ±0.02	
Raw.........	18	13.4	10	7.1	1.13	58	147	89	1.59	1.40 ±0.02	0.31 ±0.04
Toasted.....	18	13.4	12	6.4	1.06	55	120	65	1.16	1.09 ±0.04	
Raw.........	21	15.7	13	7.6	1.38	54	143	89	1.59	1.15 ±0.02	0.03 ±0.04
Toasted.....	21	15.7	11	6.7	1.25	52	131	79	1.40	1.12 ±0.04	
Raw.........	24	17.9-22.1	9	7.5	1.59	48	136	88	1.57	0.98 ±0.02	0.12 ±0.03
Toasted.....	24	17.9-22.1	12	7.0	1.56	48	124	76	1.35	0.86 ±0.02	

gain in weight per gm. of protein consumed was little changed at the lower levels for either raw or toasted gluten.   At all levels the toasted diets supported less growth, absolutely and relatively to the amount of protein eaten, than did the raw diets.   No decrease in rate of growth per gm. of protein eaten was found at the lowest levels as was found by Osborne, Mendel, and Ferry (18) in their study of lactalbumin and casein.   This is possibly due to the fact that extremely low levels of gluten were not used, although the lowest, 5.7 per cent, is comparable with the levels of lactalbumin, 6.2 and 4.9, and of casein, 9.3, at which Osborne, Mendel, and Ferry found such decreases in growth response.   However

TABLE III

Comparison of Gains per Gm. of Protein Eaten by Male and Female Rats

Type of diet	Protein level	No. of rats used	Average gain per gm. protein
	per cent		*gm.*
Raw gluten	6.1	5 male	1.39
		10 female	1.27
Toasted gluten	6.1	8 male	0.96
		9 female	0.93
Raw gluten	9.3	10 male	1.20
		8 female	1.30
Toasted gluten	9.3	10 male	1.18
		6 female	1.00
Raw gluten	11.7	2 male	1.26
		6 female	1.20
Toasted gluten	11.7	2 male	1.07
		3 female	0.91

the number of animals used by the latter investigators is too small to permit of the drawing of definite conclusions.

Effect of Sex upon Growth per Gm. of Protein—Hoagland and Snider (19) have raised the question of the effect of sex upon the rate of growth per gm. of protein eaten, and have offered some data to show that the factors obtained by this method are larger for males than females.   Our experience has not wholly borne out this contention, for although greater gains in weight are usually made by the males their greater food intake compensates therefor, so that the factor of gain per gm. of protein eaten is but little affected.   Mitchell's (25) deduction that more of the protein eaten

is used for growth by the male rats than by the females is questionable in view of the larger maintenance requirement of the males. A comparison of results found in this connection is given in Table III. Since the differences between the factors found for the two sexes appeared to be insignificant male and female animals were used in about equal numbers in all groups and the factors averaged.

Effect of Protein Level upon Protein Utilization—As shown in Table II the rate of gain per gm. of protein eaten remained little affected by changes in the protein level until the 21 per cent (crude) level was reached. On this and on the 24 per cent level a definite drop in the gain per gm. of protein was evident in both the raw and toasted gluten series. The factors for the raw gluten varied between 1.29 and 1.40 at 9, 12, 15, and 18 per cent levels and for the toasted between 0.94 and 1.09 on the same levels. Whether the high figure 1.40 for the 18 per cent raw gluten diet indicates a real maximum is difficult to discern. In any case the drop to 1.15 on the corresponding 21 per cent diet and to 0.98 on the 24 per cent diet indicate definite wastage of protein at these higher levels. The maximum figure for the toasted gluten was 1.12 at 21 per cent with a drop to 0.86 at the 24 per cent level. It is clear that the difference in the utilization of the raw and toasted proteins tends to disappear at the 21 per cent level.

The variation in food intake and corresponding growth among the rats on a given diet was great enough to require the application of statistical methods to the results. When probable errors of the differences between the factors found for raw and toasted gluten on the same levels are computed the significance of these differences up to the 21 per cent level are obvious. The variability of the growth response was found to decrease rather definitely as the protein levels rose, and the difference in value of the raw and toasted proteins largely disappeared at the 21 and 24 per cent levels, at which levels the curves of growth had begun to flatten. If these represent a *luxus* consumption of protein it is not surprising that the nutritive deficiency produced by the toasting should begin to fail to manifest itself.

The average factors for growth per gm. of protein compare well with those reported by others. Osborne and Mendel (26) found 1.3 and 1.4 for whole wheat at the 10 per cent level but with a 4

and a 10 week instead of an 8 week period of feeding. Hoagland and Snider (27) in 60 days feeding obtained a factor of 1.58 at the same level. Our figure is 1.68 for raw wheat at a somewhat lower level, 1.78 for water-cooked, and 1.12 for toasted wheat. The raw wheat gluten factors are lower, 1.29 at 10 to 12 per cent and 1.31 at 7 to 9 per cent levels. The corresponding toasted gluten diets yield factors as low as 1.04 and 0.94. The figure of 1.20 for corn obtained at approximately an 11 per cent level may be compared with 1.18 found by Maynard, Fronda, and Chen (21), at a 9 per cent level.

Our higher figures for the whole cereals may be due partly to the inclusion of 500 mg. of yeast per day in our diets, the nitrogen of which amounted to one-fourth to one-ninth the total nitrogen intake. As is mentioned later there appears to be some doubt as to the biological value of yeast proteins for the rat, since Still and Koch (28) found low values as contrasted with those found by Mitchell (23). Our data appear to support the latter since our factors for growth as well as our biological values obtained by the balance method for wheat proteins plus small amounts of yeast are at least as high as others have reported for wheat alone. Of course a supplementary effect may be involved.

Although there seems little likelihood that the heat effect is the same as that of denaturation in water solution at lower temperatures the search for the cause of this change must follow the lines suggested by experiments upon the latter phenomenon. A few feeding tests were made with toasted whole wheat and with toasted wheat gluten supplemented by various amino acids. The amino acids used were tyrosine, tryptophane, cystine, histidine, arginine, and lysine. No benefit was observed except with histidine and lysine with each of which much improved growth resulted. Further effort is being made to determine whether the injury to the protein concerns particularly these dibasic acids.

Biological Value Experiments—Another method of estimating the nutritive efficiency of food proteins, which has been largely used in recent years, is that usually identified with the name of Karl Thomas (22) who made an extensive use of it early in this century. This consists essentially of the determination of the per cent of absorbed food nitrogen which is retained in the body. The method eliminates the question of differences in digestibility by

TABLE IV

Daily Metabolic Nitrogen Data Showing Biological Values of Raw and Toasted Wheat Gluten Alone and Supplemented by Casein at 9 to 14 Per Cent Levels (Series 1 and 2)

No. of rats	No. of periods	Diet	N in diet	Average body weight	Food intake	N intake	Fecal N	Urinary N	Food N absorbed	Food N retained	Biological value	Difference between biological values of raw and toasted proteins
			per cent	gm.	gm.	mg.	mg.	mg.	mg.	mg.		
Series 1												
4	2	Raw cracked wheat.......	1.60	66	11	194	53	85	172	110	64	12
	2	Low N........	0.10	64	6	25	16	23				
4	2	Raw cracked wheat and 5 per cent casein......	2.04	72	10	216	50	94	192	130	67	—2
	2	Low N........	0.10	74	7	26	17	33				
4	2	Water-cooked cracked wheat........	1.76	77	13	267	65	103	233	156	67	15
	2	Low N........	0.10	73	7	17	15	25				
4	2	Water-cooked wheat and 5 per cent casein......	2.25	83	11	267	57	93	236	178	75	6
	2	Low N........	0.10	84	8	28	18	34				
4	2	Toasted cracked wheat.....	1.81	55	7	115	57	65	87	46	52	
	2	Low N........	0.10	50	4	24	8	21				

4	2	Toasted wheat and 5 per cent casein........	2.31	82	10	251	73	106	212	146	69	12 ±1.8
	2	Low N..............	0.10	76	6	25	18	43				
Series 2												
11	8	Raw wheat gluten.........	1.78–2.45	62	11	246	37	121	237	158	66 ±1.3	
15	8	Toasted wheat gluten......	1.69–1.98	67	10	217	44	120	198	108	54 ±1.3	
22	12	Low N..............	0.10	58	8	16	20	30				

TABLE V

Daily Metabolic Nitrogen Data Showing Biological Values of Raw and Toasted Wheat Gluten at 6 Per Cent Level (Series 3)

No. of rat	No. of periods	Diet	Average body weight	Food intake	N intake	Fecal N	Urinary N	Food N absorbed	Food N retained	Biological value	Difference between biological values of raw and toasted gluten
			gm.	gm.	mg.	mg.	mg.	mg.	mg.		
58	2	Raw gluten	243	15	185	27	94	185	142	77	
	3	Low N, egg	241	12	130	24	52				26
	2	Toasted gluten	223	11	149	41	110	132	68	51	
59	2	Raw gluten	237	11	148	18	71	146	124	85	
	3	Low N, egg	228	11	122	16	47				25
	2	Toasted gluten	217	11	143	24	88	136	82	60	
60	2	Raw gluten	232	10	137	16	86	137	111	81	
	3	Low N, egg	196	9	109	21	51				11
	2	Toasted gluten	188	9	128	24	86	124	87	70	
61	2	Raw gluten	232	12	157	22	76	152	127	83	
	3	Low N, egg	215	10	115	14	47				20
	2	Toasted gluten	212	10	135	20	94	130	82	63	
26	2	Raw gluten	222	10	128	20	68	128	112	87	
	3	Low N, egg	224	10	116	23	55				18
	2	Toasted gluten	210	10	131	20	90	131	91	69	

	No. rats	Diet									
36	2	Raw gluten	268	12	153	20	77	153	132	86	14
	3	Low N, egg	263	11	123	22	56	145	105	72	
	2	Toasted gluten	260	11	146	24	93	126	107	85	
47	2	Raw gluten	210	10	128	22	68	126	107	85	24
	3	Low N, egg	218	11	120	21	53				
	2	Toasted gluten	209	8	108	19	90	107	66	61	
48	2	Raw gluten	220	10	138	19	76	138	108	78	17
	3	Low N, egg	237	13	129	24	76				
	2	Toasted gluten	219	8	117	17	90	116	71	61	
Averages 8 rats......	16	Raw gluten (0.98 per cent N)	233	11	146	20	77	146	121	83 ±0.9	19 ±1.8
8 "	16	Toasted gluten (0.97 per cent N)	217	10	132	23	93	127	82	64 ±1.6	
8 "	24	Low N, egg (0.73 per cent N)	227	11	125	20	51				

the determination of so called "metabolic" nitrogen of the feces on low nitrogen intake and the correction of fecal nitrogen output

TABLE VI

Daily Metabolic Nitrogen Data Showing Biological Values of Raw and Toasted Casein at 8 Per Cent Level

No. of rat	No. of periods	Average body weight	Condition of casein	Food intake, 1.3 per cent N	N intake	Fecal N	Urinary N	Food N absorbed	Food N retained	Biological values	Difference between biological values of raw and toasted casein
		gm.		gm.	mg.	mg.	mg.	mg.	mg.		
26	2	216	Raw	12	194	45	101	179	130	72	
	2	220	Toasted	10	165	63	118	125	60	48	24
36	2	274	Raw	12	193	44	115	172	114	67	
	2	262	Toasted	9	148	46	116	119	57	48	19
47	2	220	Raw	9	159	37	107	140	86	61	
	2	220	Toasted	8	148	35	118	130	65	50	11
48	2	230	Raw	10	169	41	105	148	91	61	
	2	229	Toasted	11	179	41	123	159	83	52	9
58	2	233	Raw	12	191	35	94	180	133	74	
	2	233	Toasted	11	186	42	117	166	98	59	15
59	2	196	Raw	8	143	35	96	125	69	55	
	2	205	Toasted	12	192	40	118	171	93	54	1
60	2	185	Raw	8	150	35	90	133	91	68	
	2	193	Toasted	10	173	41	108	155	97	63	5
61	2	192	Raw	10	176	38	93	151	101	67	
	2	198	Toasted	9	160	40	115	132	61	46	21
Averages 8 rats...	16	218	Raw	10	172	39	100	151	100	66 ±1.5	
8 " ...	16	222	Toasted	10	169	43	117	144	77	53 ±1.4	13 ±2.0
8 " ...	24	227	Low N, egg	11	125	20	51				

thereby.   The endogenous nitrogen output is similarly determined and urinary nitrogen corrected accordingly.   Mitchell (29) has

recently modified this procedure for use with rats and has discussed its value and limitations in considerable detail.

This method was used upon groups of young rats, two in a cage, a different pair being used for each experiment, and later upon single mature rats, the same animals being used for toasted and raw protein diets in succession. In the former set of tests rats 35 days old were used. In all cases the animals were kept on the diet 7 days before collection of excreta was begun and collection periods were 4 or 5 days in length. One nitrogen-low or endogenous period was obtained with the young rats used in Series 1 and 2 (Table IV) on wheat gluten and three with each of the older rats used in Series 3 on wheat gluten (Table V) and in the casein tests (Table VI). The diets were the same as those used for the preliminary growth experiments, 95 per cent cereal, and for the wheat gluten tests, 12 per cent and 6 per cent levels being selected in the latter case. Casein, raw and toasted as was the gluten, was used at the 8 per cent level also with the older rats.

The diets given during the low nitrogen periods were of two kinds. The younger rats used in Series 1 and 2 on wheat gluten were given a protein-free diet made up of:

	per cent
Corn-starch	88
Agar	4
Salt mixture	3
Lard	5

Dried yeast, 500 mg., or yeast concentrate, 50 mg., and cod liver oil, 25 mg., were also given separately each day to each rat. In Series 3 on gluten and in the casein tests a modified form of the low egg diet recommended by Mitchell and Carman (30) was used instead. This was made up of:

	per cent
Dried whole egg	8.5
Agar	4
Sucrose	10
Wheat starch	63.5
Salt mixture	4
Lard	10

Yeast and cod liver oil were fed separately as before. The salt mixture used in all these experiments is that of Osborne and Men-

del (17). The experimental data were corrected for metabolic or body nitrogen in the feces by average figures for fecal nitrogen per gm. of food consumed found in the protein-free or low egg periods, as was the urinary nitrogen for endogenous or body nitrogen per 100 gm. of body weight found in the same periods.

Effect of Yeast Nitrogen—The yeast nitrogen was included in the total nitrogen intake. This amounted to 10 to 19 per cent of the total nitrogen intake in Series 1 (Table IV) with the whole wheat diets, except that it was 25 and 31 per cent of the toasted wheat diets. In Series 2 (Table IV) for the most part 50 mg. of yeast concentrate were used instead of 500 mg. of the dried yeast so that the per cent of total nitrogen intake from this source was only 4 to 6. In Series 3 (Table V) the average per cent of nitrogen from yeast was 21 in the raw and 24 in the toasted gluten diets. In the casein experiments the figure was 22 for both raw and heated diets.

The proportion of yeast nitrogen in the endogenous series was of course higher, 25 to 30 per cent in the low egg diet and 50 to 70 in the protein-free diets. According to Still and Koch (28) yeast protein is of rather low biological value, 21 to 26, in diets fed to rats and is apparently not an advantageous supplement to casein since diets containing one-half of the nitrogen from yeast and one-half from casein showed only the expected biological values. Whether yeast nitrogen supplements gluten better than it does casein has not been shown. However the proportion of yeast nitrogen is comparable in all raw and toasted diets with the possible exception of the two experiments on toasted wheat in Table IV. Due to low food intake the yeast proportion is higher in these cases than in the corresponding raw wheat tests. At a maintenance level, 5 per cent, yeast protein was found by Mitchell (23) to yield a biological value of 85, a figure comparable with our best average obtained with raw gluten at a somewhat similar level (Table V). It cannot be assumed therefore that the presence of the small amount of yeast nitrogen in our experimental diets exerted any measurable influence on the biological values obtained.

The methods of caging and of making collections of excreta have been described previously (31). In Series 1 and 2 on wheat proteins two young rats were caged together, their intake and output being considered collectively. Moreover a different pair was

used for every test.   This procedure was adopted partly because very young animals could thus be utilized and partly to make more crucial the comparison of the raw and toasted proteins.   The variation between pairs of figures obtained in this way however appears to be no greater than those obtained by repetitions of the same diet with separately caged mature rats.

As seen in Table IV the raw whole wheat gave a protein biological value of 64, the water-cooked wheat 67, and the toasted wheat 52.   The protein levels used were 9 and 10 per cent.   When 5 per cent dry casein was substituted for a corresponding amount of the starch in these diets the figures obtained at the 12 to 14 per cent level were 67, 75, and 69.   The supplementing effect of the casein is obviously more valuable in the toasted diets.   The actual values obtained may be compared with that reported by Mitchell and Carman (32) for raw wheat, 67, at an 8 per cent level and that by Mattill (33), 72, at a 5 per cent level.   In the same table are shown similar results for the wheat gluten diets used in Series 2, the average being 66 for the raw and 54 for the toasted gluten fed at approximately 12 per cent levels.   The agreement between these results and those found with the whole wheat is satisfactory. The probable error of the values found in Series 2 was in both cases 1.3 and the difference of the means is more than 6 times the size of its probable error.

For the determinations recorded in Table V mature rats were used with a low level of protein, about 6 per cent, and correspondingly higher biological values obtained, 83 for the raw and 64 for the toasted gluten.   This is a higher value than has hitherto been reported for wheat but is the first obtained at so low a level upon wheat gluten rather than whole wheat.   The probable error of the sixteen values found for the raw gluten is 0.9 and for the toasted 1.6.   The difference of the means is more than 10 times its probable error.

In Table VI are given the results of one set of experiments upon casein, raw and toasted.   Commercial casein was used at an 8 per cent level with mature rats.   Surprisingly enough the biological values obtained were only 66 and 53, no better than the figures found on considerably higher levels of gluten (Table IV). Mitchell (23) using a 5 per cent level of raw casein found an average value for sixteen determinations of 71.   This is in good agree-

ment with our figure of 66 at the slightly higher level. Again the difference between the means of the biological values for raw and toasted casein is more than 6 times its probable error. The significance of all these differences would seem therefore to be reasonably established.

Digestibilities of Raw and Toasted Proteins—It is interesting to observe the considerably better absorption of the gluten than of the casein nitrogen. The digestibilities of all the proteins studied are summarized in Table VII in which it is seen that the nitrogen of the whole wheat preparations is less completely absorbed than is that of the gluten, 88 and 95, in comparable animals, and

TABLE VII

Digestibilities of Raw and Toasted Gluten and Casein

No. of rats	Average body weight	Type of protein	Level of protein in diet	Digestibility of protein (corrected)	Biological value
	gm.		per cent	per cent	
4	59	Raw whole wheat	12	88	63
4	54	Toasted whole wheat	11	73	52
4	66	Water-cooked whole wheat	12	87	66
11	62	Raw wheat gluten	10–14	95	65
15	67	Toasted wheat gluten	10–12	90	55
8	233	Raw wheat gluten	5.6	98	83
8	217	Toasted wheat gluten	5.5	96	64
8	218	Raw casein	8	88	66
8	222	Toasted casein	8	85	53

that the older rats used in Series 3 digested the gluten more completely than did the young ones, used in Series 2. The raw and toasted casein were only 88 and 85 per cent digestible as compared with 98 and 96 per cent for the gluten. The toasted whole wheat used in Series 1 averaged 15 per cent less absorbability than the raw wheat, but the toasted wheat gluten was only 5 per cent less digestible by the young rats and 2 per cent less by the mature rats than the raw gluten.

In the latter cases it is evident that the disparity in nutritive value between the toasted and raw products must lie chiefly in differences in the usefulness of the amino acid mixtures absorbed. These differences, which range from 10 to 19 per cent, are clearly

significant of some change in the amino acid content or availability of the proteins produced by dry heating at 150 to 200° for 30 to 45 minutes.

SUMMARY

1. Preliminary growth experiments with young rats showed that the protein of cereals subjected to dry heat or toasting at approximately 200° for 45 minutes or to similar procedures during manufacture is not well utilized for growth.

Cooking with water had but little similar effect and caramel formation during toasting was found to account for only a small part of the injury.

The addition of 5 per cent casein to these toasted diets very nearly made up the discrepancy between the latter and correspondingly supplemented raw diets, thus indicating that the deficiency observed lay in the protein fraction of the toasted diet.

2. When young rats were fed diets containing raw and toasted (heated at 150° for 30 minutes) wheat gluten as source of protein at 9, 12, 15, 18, 21, and 24 per cent levels (crude protein) in a diet otherwise adequate the growth in 56 days per gm. of protein eaten was found to be 1.29 to 1.40 for raw gluten at all levels up to 18 per cent and to fall to 1.15 and 0.98 at 21 and 24 per cent. The corresponding figures for the toasted gluten were 0.94 to 1.09, 1.12 and 0.86. The maximum growth rate on both raw and toasted diets was seen at the 18 per cent level, but the toasted gluten supported less growth than the raw both absolutely and relatively to the amount eaten at all levels.

3. The biological values of raw, water-cooked, and toasted whole wheat protein alone and supplemented by 5 per cent casein as determined by nitrogen balance on young rats according to the modified Mitchell method were found to be 64, 67, and 52 for wheat alone and 67, 75, and 69 when supplemented by the casein, and in a similar comparison of biological values of raw and toasted wheat gluten at an approximately 12 per cent level the figures obtained were 66 and 54.

4. When large mature rats were used for a similar study of raw and toasted wheat gluten at a 6 per cent level, the values found were 83 and 64 and in another test with raw and toasted casein at an 8 per cent level the biological values found were 66 and 53.

In all cases the differences between the biological values of raw

and toasted proteins were found to be more than 6 times as great as their probable errors.

5. The *digestibility of the toasted proteins was but little different from that of the raw, particularly in the older animals, and the unexplainable loss of nitrogen occurred chiefly in the urine,* indicating that the change produced by the heat treatment lies probably in the assortment or availability of the amino acids absorbed.

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